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(54) Title: HUMAN CYCLIN E

(57) Abstract

Nucleic acid molecules capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 1 and 1185 of the human cyclin E cDNA sequence shown in the figure. Polypeptides encoded by such nucleic acid molecules, and immunologic binding partners directed to such polypeptides.

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HUMAN CYCLIN E

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Field of the Invention

This invention relates to genetic engineering involving recombinant DNA technology, and particularly to the identification of a nucleotide sequence encoding human cyclin E that controls the rate of cell growth by controlling progression at G1 phase of the cell cycle and entry into the S phase.

Background of the Invention

A major goal in studying the growth and differentiation of higher eukaryotic cells is to describe in biochemical terms the pathways, enzymes, and cofactors that regulate progression through the cell cycle, and in particular through the transitions from G1 phase into S phase, and from G2 phase into M phase. Proteins, now known as cyclins, were described in fertilized sea urchin and clam eggs as members of a small number of proteins whose synthesis was greatly stimulated following fertilization (in the appended Citations: Evans, et. al., 1983) and whose levels decreased at each mitosis. Cyclin A (Swenson et al., 1986) and cyclin B (Pines and Hunt, 1987), were discovered to periodically accumulate in mitotic cells, and thus a role in the mitotic process was considered possible (Evans et al., 1983) even though the biochemical basis was unclear. Results of genetic and biochemical analysis now support a role for certain cyclins in meiosis and mitosis. Microinjection of clam or sea urchin cyclin B1 mRNA into *Xenopus* oocytes (Pines and Hunt, 1987);Westendorf et al., 1989) is reportedly sufficient to drive the cell through meiosis I and II, and cyclin B may be the only protein whose synthesis is required for each mitotic cycle in early *Xenopus*

embryos (Murray and Kirschner, 1989). Conversely, destruction of cyclin B1 and B2 mRNA may cause fertilized *Xenopus* eggs to arrest after DNA replication but before mitosis (Minshull et al., 1989). Besides *Xenopus*, in the yeasts *S. pombe* and *S. cerevisae* cyclin B reportedly plays a role in regulating transit through mitosis (Hagan et al., 1988; Ghiara et al., 1991; Surana et al., 1991; Booher and Beach, 1987; Booher et al., 1989; Hagan et al., 1988; Ghiara et al., 1991; Surana et al., 1991) by exerting mitotic control over activation of a p34 CDC2 protein kinase (reviewed in Nurse, 1990; Cross et al., 1989). In the latter case, CDC2 kinase is reportedly not catalytically active as a monomer, but following binding to the cyclin B and a series of phosphorylations and dephosphorylation steps, the kinase activity is generated (Simanis and Nurse, 1986; Draetta and Beach, 1988; Pondaven et al., 1990; Solomon et al., 1990; Gould and Nurse, 1989; Enoch and Nurse, 1990; Solomon et al., 1992).

Cyclin B-dependent activation of a p34 CDC2 kinase may also be necessary to initiate mitosis in certain somatic cells (Nurse, 1990; Cross, 1989; Maller et al., 1991), but activation alone may not be the only event required (Lamb et al., 1990; Osmani et al., 1991; Amon et al., 1992; Sorger et al., 1992). *S. cerevisiae* apparently has a CDC2 homologue termed CDC28. The *CDC2* and *CDC28* gene products appear to be structurally similar (Lorincz & Reed, 1984; Hindley & Phear, 1984) and functionally homologous (Beach et al., 1982; Booher & Beach 1987). They encode a serine/threonine protein kinase that is the homolog of the 34 kDa protein kinase in vertebrate and invertebrate mitosis promoting factor (MPF; Lee & Nurse, 1987; Arion et al., 1988; Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1988). CDC28 may require different cyclins for the cell cycle transitions at G2/M and at G1/S: namely, at G2/M CDC28 reportedly binds to and is activated by B-type cyclins (Ghiara et al., 1991; Surana et al., 1991), while at G1/S CDC28 is reportedly activated by CLN-type cyclins, (i.e., CLN1, CLN2 and CLN3; Sudbery et al., 1980; Nash et al., 1988; Cross, 1988, 1990; Hadwiger et al., 1989; Richardson et al., 1989; Wittenberg et al., 1990).

CLN1 and CLN2 cyclins are periodically expressed during the cell cycle, peaking in abundance at the G1/S transition point (Wittenberg et al., 1990; Cross and Tinkelenberg, 1991) and accumulation of the CLN proteins in yeast cells may be rate limiting for the transition from G1 into S phase of the cell cycle.

For the purposes of the present disclosure, the term "CDC protein kinase" is used synonymously with the recently adopted "cell division kinase (CDK)" nomenclature.

The p34 CDC2 kinase activity apparently oscillates during the cell cycle (Mendenhall et al., 1987; Draetta & Beach; 1988; Labbe et al., 1989b; Moreno et al., 1989; Pines & Hunter, 1990), and this oscillation of activity is not attributable to variations in the amount of the *CDC2* gene product present in cells (Durkacz et al., 1986; Simanis & Nurse, 1986; Draetta & Beach, 1988). Rather, CDC2 kinase activity appears to be influenced by interactions of the kinase with other proteins, including (as discussed above) the cyclins (Rosenthal et al, 1980; Evans et al., 1983; Swenson et al., 1986; Draetta et al., 1989; Meijer et al., 1989; Minshull et al., 1989; Murray & Kirschner, 1989; Labbe et al., 1989a; Soloman et al., 1990; Gautier et al., 1990; reviewed in Murray & Kirschner, 1989; Hunt, 1989). Apparently an association between a p34 CDC2 protein and a B-type cyclin is necessary for the activation of the p34 kinase at the onset of mitosis in a wide variety of organisms including yeast (Booher & Beach, 1987; Hagan et al., 1988; Moreno et al., 1989; Soloman et al., 1988; Booher et al., 1989; Surana et al., 1991; Ghiara et al., 1991) and humans (Draetta & Beach, 1988; Pines & Hunter, 1989; Riabowol et al., 1989).

In budding yeasts a major control decision point in cell proliferation reportedly occurs during G1, i.e., at a point termed START, where entry of cells into S phase is restricted until certain conditions have been satisfied (Hartwell, 1974). The START transition appears to require a *CDC28* or *cdc2* gene product (Hartwell et al., 1973, 1974; Nurse & Bisset, 1981), but the biochemical pathways that activate CDC28 at START are not completely understood. The latter pathways may involve the CLN1, CLN2 and CLN3 cyclins and activation of CDC28 because cells deficient in all three CLN proteins arrest at START; and although they continue to grow they are unable to enter S phase (Sudbery et al., 1980; Nash et al., 1988; Cross, 1988, 1990; Hadwiger et al., 1989; Richardson et al., 1989; Wittenberg et al., 1990). CLN2, and probably CLN1 and CLN3, may form complexes with CDC28 kinase prior to or at START (Wittenberg et al., 1990). The CLN1 and CLN2 oscillates during the cell cycle, but maximal levels are reportedly observed in late G1 (i.e., rather than late G2; Wittenberg et al., 1990).

Little is currently known about the biochemical pathways that control the start of DNA synthesis in higher eukaryotic cells or the extent to which these pathways resemble those in yeast. However, in human cells (as in budding yeast) the predominant mode of control of cell proliferation appears to occur during the G1 phase of the cell cycle (Zetterberg & Larson, 1985; Zetterberg, 1990). The kinetics of passage through G1 in mammalian cells suggest a single decision point, termed the "restriction point", that regulates commitment of a cell to initiate DNA synthesis

(Pardee, 1974). Prior to the restriction point, progress through G1 is sensitive to the growth state of the cell (e.g., reducing the rate of protein synthesis or removing a growth factor apparently may delay entry into S phase and can even cause cell cycle arrest), however, after the restriction point the cell cycle becomes substantially less responsive to these signals (reviewed in Pardee, 1989). Unlike yeasts, CDC2 cyclin appears to be diversified into a small protein family in mammalian cells (Paris et al, 1991; Elledge and Spotswood, 1991; Tsai et al., 1991; Koff et al., 1991) and CDC2/28 activities may also be split among several different kinase family members (Fang and Newport, 1991). Certain cyclins may have roles in G1 regulation in higher eukaryotes similar to those reported in yeast. For example, cyclin A synthesis reportedly begins late in G1 and it may activate both p34 CDC2 and certain related p33 CDK2 kinases (Giordano et al, 1989; Pines and Hunter, 1990; Marraccino et al., 1992; Tsai et al., 1991). Inhibition of cyclin A function may also reportedly block a START-like function of S phase in certain cells (Girard et al., 1991) and cyclin A reportedly is able to associate with certain transforming and growth suppressing factors (Hunter and Pines, 1991). However, despite these apparent results supporting a role for cyclin A in regulating a START-like function in higher eukaryotes, there are also some reasons to doubt that cyclin A is functionally homologous with budding yeast CLN proteins. Several laboratories have recently identified two novel cyclins in mammalian cells that are not present in yeasts, i.e., cyclin C and cyclin D. The *cyclin D* gene was reported as a gene induced by CSF-1 in murine macrophages in late G1 (Matshushime et al., 1991) and the gene may have a chromosomal location at a breakpoint subject to possible rearrangement in human parathyroid tumor (Motokura et al., 1991). Cyclin C, as well as cyclin D, have also been reportedly identified in human and *Drosophila* cDNA libraries by screening for genes capable of complementing mutations in *S. cerevisae CLN* genes (Laheu et al, 1991; Lew et al., 1991; Leopold and OFarrell, 1991; Xiong et al., 1991). While the results are consistent with G1 functions for cyclin C and cyclin D, cyclin B (a mitotic cyclin) was also found to be capable of rescuing the latter *S. cerevisae* CLN mutants, indicating that yeast complementation assays may not necessarily identify cyclins that perform similar functions in higher eukaryotic cells.

The similarities between the restriction point in mammalian cells and START in yeast has suggested a possible role for a p34 CDC2 kinase. In support of this hypothesis, a human *CDC2* gene has been found that may be able to substitute for the activity of an *S. pombe cdc2* gene in both its G1/S and G2/M roles (Lee & Nurse, 1987). Also, cell fusion experiments offer circumstantial evidence in support of the

hypothesis (Rao & Johnson, 1970) since a diffusible trans-acting factor is reportedly involved in activation of DNA synthesis when S phase cells were fused to G1 cells. However, the relationship between the latter S phase activator and the p34 CDC2 kinase remains unclear. Recently cyclin-CDC2 complexes have reportedly been isolated from human S phase cells and shown to be active in inducing SV40-DNA replication when they were added to extracts of G1 cells (D'Urso et al., 1990). Antisense oligonucleotides directed against the human *CDC2* mRNA are reportedly inhibitory for human PHA-activated T cells at entry to S phase (Furakawa et al., 1990). In other higher eukaryotic cells it has been reported that depletion of CDC2 protein from *Xenopus* extracts can block DNA replication (Blow & Nurse, 1990). Despite recent suggestive reports, the pathway that activates p34 kinase during the G1 phase of the human cell cycle is not currently understood.

By analogy with the CLN-dependent activation of CDC28 at START in yeast, it is possible that specific G1 cyclins may play a role in regulating the human p34 kinase during the G1 to S phase transition. To test this idea experiments were conducted herein to determine whether human cells contain specific cyclins that can replace the yeast *S. cerevisae* CLN proteins. This assay identified a new human cyclin, cyclin E.

Summary of the Invention

The invention provides isolated nucleic acid molecules capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 1 and 1185 of the human *cyclin E* cDNA sequence shown in FIGURE 2. Such nucleic acid molecules preferably encode cyclin E polypeptides capable of binding and activating a cell division kinase (e.g., CDC2, CDC28, CDK2-XL, CDC2-HS, and CDK2-HS). The cyclin E polypeptide is typically also capable of shortening the G1 phase of the cell cycle. The invention also provides polypeptides encoded by the aforesaid nucleic acid molecules, and immunologic binding partners capable of specifically binding the polypeptides.

Cyclin E functions specifically during the late G1 and early S phases of the cell cycle by binding and activating a CDC2 related protein kinase, CDK2. The levels of the cyclin E/CDK2 polypeptide complexes are cell cycle-regulated, and peak in abundance in late G1 phase of the cell cycle. Constitutive expression of cyclin E in cells is alone sufficient to shorten the G1 phase of the cell cycle and promote cell growth. Increasing or decreasing the levels of cyclin E in a cell increase or decreases cell growth, respectively. Detecting the levels of cyclin E in cells such as tumor cells

may provide information on their rate of growth. Rearrangement of the location of *cyclin E* at chromosomal breakpoints may change the rate of cell proliferation.

Brief Description of the Drawings

FIGURE 1 shows complementation of the triple *cln* deletion by human *cyclin E*: *S. cerevisae* strain 589-5 contains deletions of the chromosomal *CLN1*, *-2*, and *-3* genes and contains the *GAL1-CLN3* gene on a multicopy episome. It was transformed with the *pADNS* expression vector or the *pADNS* vector containing a human cyclin E cDNA. Transformants, and the parental strain, were streaked on galactose and glucose and grown for 3 days at 30°C.

FIGURE 2 (SEQ. ID. NOS. 1-2) shows the sequence of cyclin E: DNA and predicted protein sequence of the cyclin E cDNA that complemented the triple *cln* deletion.

FIGURE 3 shows alignment of the protein sequences of human cyclins A, B, and E: Protein sequences of cyclins A, B, and E were aligned to maximize homology. Boxes indicate identical amino acids. Amino acid shared by all three cyclins are shown in bold type above the three sequences. The area highlighted by double bold lines is a domain highly conserved among all known cyclins (the "cyclin box"). The domain highlighted by single bold lines is the mitotic destruction motif shared by all A- and B-type cyclins.

FIGURE 4 shows efficiency of rescue of the triple *cln* deficiency by human cyclins E and B in *CDC28* or *cdc28-13* strains: All strains tested were *cln1-cln2-cln3-* (*pGAL-CLN3*); these strains were transformed with the indicated vector plasmids, *pADNS* or *pADANS*, or the vector plasmids containing either human cyclin E or B by selecting for leucine prototrophy. The vector *pADNS* uses the yeast *ADH* promoter for expression of the cDNA. The vector *pADANS* is identical to *pADNS* except that the expressed protein is fused at its amino terminus to the first 10 amino acids of the ADH protein. No transformants could be obtained with the plasmid *pADNS-CYC B*, suggesting it was lethal. The number of viable colonies in an inoculum of stationary phase culture in galactose was determined by serial dilution followed by 4-5 days growth on both galactose- and glucose-containing medium at 30°C. The plating efficiency is defined as the number of glucose-viable colonies divided by the number of galactose-viable colonies. Only colonies resulting from plasmid bearing cells are used in the calculation. Both YC and YEP media were used with comparable results. The vector and *pADNS-CYC E* values were determined in four experiments using two different pairs of *CDC*$^+$ and *cdc28-13* strains (one *CDC*$^+$ and one *cdc28-13* strain tested in parallel in each experiment). The

pADANS-CYC E values come from a single experiment. The cyclin B values were determined in two experiments, both with the same pair of CDC^+ and *cdc28-13* strains. All strains were isogenic. The ranges of values for the CDC^+ strains were: for *pADNS-CYC E*, 0.12-0.4; for *pADANS-CYC B*, 0.19-0.33; for the *cdc28-13* strain *pADNS-CYC E*, 0.0006-0.008; and for *pADANS-CYC B*, 0.2-0.4. With both cyclin E plasmids the colony sizes for *cdc28-13* strains on glucose medium were significantly smaller than colony sizes for CDC^+ strains; for the cyclin B plasmid the colony sizes were similar in the CDC^+ and *cdc28-13* strains.

FIGURE 5 shows construction of a yeast strain in which *CDC28* is defective for START but not G2/M: Yeast strain 1238-14C-cycE has the following relevant genotype: $cln1^- cln2^- cln3^- cdc28^{13}$ (*pADH-cycE-TRP1,pGAL-CLN3-URA3*). The putative cyclin-cdc28-13 complexes which control the G1/S and G2/M transitions in this strain are indicated. Shown are cyclin-cdc28-13 complexes that form on either galactose or glucose and the functional activity of those complexes at either 30° or 38°C. CLB is the nomenclature used to designate the *S. cerevisae* homologs of the B-type cyclins. On glucose at 30°C this strain is defective for START but not G2/M.

FIGURE 6 shows efficiency of rescue of the triple *cln* deficiency in a *cdc28-13* strain by human cyclin E in conjunction with human *CDC2* or human *CDK2*: A strain of genotype $cln1^-$ $cln2^-$ $cln3^-$ *cdc28-13* (pGAL-CLN1/URA3) was cotransformed with either *pMAC-TRP1-CYC E* and *pADNS-LEU2-CDC2-HS* or with *pMAC-TRP1-CYC E* and *pADNS-LEU2-CDK2-HS*. Transformants were selected for leucine, tryptophan, and uracil prototrophy on galactose. Two independent transformants were grown nonselectively overnight, and plasmid loss events were identified following colony purification. This resulted in the generation of isogenic sets of strains, either containing both cyclin E and *CDC2-HS* (or *CDK2-HS*) or either gene alone. Two such sets were generated for each cotransformation. The twelve strains were tested in the quantitative plating assay described above (see legend to FIGURE 4) except that plating efficiencies were measured both at 30° and 38°C. Strains containing the same plasmid combinations behaved very similarly, and their data are pooled in the table. Note that unlike cyclin E plasmids used in the experiments in FIGURE 4, the *pMAC-TRP1-CYC E* plasmid used in these experiments gives essentially no rescue of the $cln1^-$ $cln2^-$ $cln3^-$ *cdc29-13* strain.

FIGURE 7 shows cyclin E can bind and activate the p34 cdc2 kinase in extracts from human G1 cells: Extracts from newborn MANCA human G1 cells were mixed with GT-cyclin E-Sepharose beads, GT-Sepharose, p13-Sepharose, or blank Sepharose beads. In panel A, the bound proteins were immunoblotted with

anti-peptide antiserum against the carboxy terminus of human CDC2. In lanes labeled "+" the Sepharose beads had been incubated with the human G1 extract. In lanes labeled "-" mock incubations with buffer were performed. The arrow indicates the bound 34 kDa protein that reacts with the C-terminal antibodies. In panel B, the beads were assayed for histone H1 kinase activity. Arrows indicate the mobility of histone H1 and GT-cyclin E fusion protein markers. In the lane labeled "cycE-IP" the proteins associated with the GT-cyclin E-Sepharose beads were released with free glutathione and immunoprecipitated with a cyclin E antiserum. In panel C, the proteins released from either the GT-Sepharose or GT-cyclin E-Sepharose by free glutathione were immunoprecipitated with an affinity-purified C-terminus-specific p34 CDC2 anti-peptide antiserum. The immunoprecipitates were tested for H1 kinase activity.

FIGURE 8 shows immunoprecipitation of an H1 kinase activity from HeLa cells using anti-cyclin E antibodies: In panel A, an antiserum raised in rabbits against the GT-cyclin E fusion protein was used to immunoprecipitate *in vitro* translated human cyclins E, A, and B. Lanes 1-3: *in vitro* translation products of human cyclins E, A, and B respectively. Lanes 4-6: the immunoprecipitates using the cyclin E antiserum of human cyclins E, A, and B respectively. In panel B, extracts from exponentially growing HeLa cells were immunoprecipitated with normal rabbit serum, anti-cyclin E serum, and anti-cyclin A serum. The immunoprecipitates were tested for H1 kinase activity. Note the autophosphorylation of a 45 kDa protein within the cyclin E immunoprecipitates. This protein comigrates with cyclin E protein produced by *in vitro* transcription/translation of the cyclin E cDNA.

FIGURE 9 shows differential levels of cyclin E-kinase complexes in different subpopulations of exponentially growing MANCA cells that were fractionated by centrifugal elutriation into different stages of the cell cycle, as described in Example 8.

FIGURE 9A shows graphically the DNA content (ordinate) of exponentially growing MANCA cells measured cytofluorimetrically in different elutriated fractions (abscissa).

FIGURE 9B shows graphically the level of cyclin E H1 histone kinase activity in the elutriated fractions of FIGURE 9A.

FIGURE 9C shows graphically the level of cyclin A H1 histone kinase activity in the elutriated fractions of cells of FIGURE 9A.

FIGURE 9D shows graphically the DNA content (ordinate) of MANCA cells in different elutriated cell fractions released into the G1 phase of the cell cycle for 3, 4, 5, 6, or 7 hours after nocodazole-induced metaphase.

FIGURE 9E shows a bar graph depicting the levels of cyclin A and cyclin E associated H1 histone kinase activity.

FIGURE 10, discussed in Example 8, shows the levels of cyclin E, as determined by measuring H1 kinase activity, in immunoprecipitates of quiescent, growing, or differentiating, rat 208F and PC-12 cells.

FIGURE 10A shows an autoradiogram of ^{32}P-labeled H1 histone. The phosphorylation of H1 histone was catalyzed by immunoprecipitates of 208F cells grown in 10% or 0.1% calf serum. The level of cyclin E-associated kinase activity was markedly reduced in quiescent (0.1% CS) cells as compared to growing cells (10% CS).

FIGURE 10B shows an autoradiogram of ^{32}P-labeled H1 histone. The phosphorylation of H1 histone catalyzed by immunoprecipitates was determined for PC-12 cells grown in the presence or absence of nerve growth factor. The level of cyclin E-associated kinase activity was markedly reduced in differentiated (quiescent) cells (-NGF) as compared to rapidly proliferating cells (+NGF).

FIGURE 11, discussed in Example 9, shows the results of studies designed to investigate increased levels of constitutively expressed cyclin E in Rat-1 cells transduced with either a retroviral vector encoding *cyclin E* (LXSN-*cyclin E*), or the LXSN vector as a negative control.

FIGURE 11A(a) shows an autoradiograph of a Western immunoblot of Rat-1 cellular lysates transduced with either LXSN-*cyclin E* (lane 2) or, as a negative control, the LXSN vector alone (lane 1). Increased levels of cyclin E, as measured by histone H1 kinase activity, were visible in LXSN-*Cyclin E* transduced cells relative to the control.

FIGURE 11A(b) shows an autoradiogram of ^{32}P-labeled histone H1 catalyzed by cyclin E-associated kinase in cellular immunoprecipitates of *LXSN-cyclin E* transduced Rat-1 cells (lane 2) or *LXSN* transduced control Rat-1 cells (lane 1). Increased *cyclin E* expression was visible in cellular immunoprecipitates of LXSN-*cyclin E* transduced Rat-1.

FIGURE 11B graphically presents the results of flow cytometric measurement of nuclear DNA content in Rat-1 cells transduced with either LXSN (Rat-1/control) or the *LXSN-cyclin E* retroviral vector (Rat-1/cyclin E), as well as the calculated fraction of the cells in each cell subpopulation that was in the G1, S, or G2/M phases of the cell cycle.

FIGURE 11C graphically presents the results of immunochemical detection of BrdU (5-bromodeoxyuridine) incorporation into nuclear DNA of Rat-1 cells

transduced with LXSN-*cyclin E* (solid diamonds) or LXSN (open squares) as a function of time after removing a mitotic block. Only cells synthesizing DNA (S-phase cells) incorporate BrdU and score positive in this assay, and so the assay measures the rate at which cells transition from the conclusion of one mitosis into DNA synthesis for the next round of mitosis. The results show that LXSN-*cyclin E* transduced cells transition from mitosis into S-phase more rapidly than LXSN-transduced control cells.

FIGURE 12, discussed in Example 10, shows the results of a study analyzing proteins that are associated with cyclin E in exponentially growing MANCA cells at different stages in the cell cycle. The cyclin E-associated proteins were purified by immunoprecipitation with anti-cyclin E antibodies and SDS-PAGE.

FIGURE 13, discussed in Example 10, shows autoradiograms of Western immunoblots prepared following SDS-PAGE of immunoprecipitates prepared from MANCA cell extracts that were immunoprecipitated with anti-CDC2 ("αCDC2"), anti-CDK2 ("αCDK2"), or control serum ("--"). The results show the specificity of the anti-CDC2 and anti-CDK2 antibodies used in FIGURES 9-12 above. The immunoblots were visualized by reacting the gels with anti-CDC-2 or anti-CDK2 followed by ^{125}I-Protein A. The immunoprecipitates in lanes 1 and 2 were prepared using whole cell extracts; those in lane 3 and 4 were control extracts that were precleared of CDC2; those in lanes 5 and 6 were precleared of CDK2; and lane 7 was an extract of MANCA cells arrested at the G1/S boundary. The positions migrated by protein molecular weight standards is as indicated.

FIGURE 14, described in Example 10, shows Western immunoblots detecting complexes of CDC2 and CDK2 with cyclin E in exponentially growing MANCA cells and cells arrested at the G1/S boundary with aphidicolin by methods described in Example 10.

FIGURE 14A shows an immunoblot of purified cyclin E:CDC2 complexes separated into its two constituent proteins on SDS-PAGE and visualized by immunoblotting with antibodies specific for the C-terminus of human p34 CDC2. Lanes 1 and 8 were negative control samples prepared from an incubation of cell extracts with αPI; lanes 2 and 7 were negative controls from an incubation of cell extracts with Sepharose beads (SEPH); lanes 3 and 6 show cyclin E:CDC2 complexes purified by affinity chromatography or anti-p34 CDC2 Sepharose; lanes 4 and 5 show cyclin E:CDC2 complexes purified by affinity chromatography on anti-cyclin E-Sepharose; lanes 9-11 labeled "--" are negative control samples prepared in a manner identical to lanes 1-8, but without cell extract.

FIGURE 14B shows an immunoblot of purified cyclin E:CDK2 complex separated into its two constituent proteins and CDK2 visualized by immunoblotting with antibodies specific for the C-terminus of human CDK2. The abbreviations used are as indicated in FIGURE 14A. The location on SDS-PAGE of CDK2 in nonaffinity-purified SDS-PAGE purified cell extract ("EX") from cells at the G1/S boundary is shown in lane 8.

FIGURE 15, described in Examples 11-12, shows the results of studies designed to determine the level of expression of cyclin E and abundance of the cyclin E:CDK2 complex at different stages in the cell cycle.

FIGURE 15A shows graphically the DNA content (ordinate) in various fractions of elutriated MANCA cells as determined by flow cytometry of propidium iodide stained nuclei.

FIGURE 15B1 shows graphically the ^{125}I-Protein A CPM bound by the CDK2 polypeptide bands immunoblotted in FIGURE 15B2.

FIGURE 15B2 shows Western immunoblots measuring the level of expression of cyclin E in each elutriated subpopulation of cells (from FIGURE 15A) as determined by immunoaffinity purification of cyclin E:CDK2 complexes with anti-cyclin E-Sepharose followed by SDS-PAGE, and visualization of the proteins in the complex by Western immunoblot analysis using anti-CDK2, ^{125}I-Protein A, and autoradiography.

FIGURE 15B3 shows graphically the ^{125}I-Protein A CPM bound by the cyclin E ("cycE") polypeptide bands immunoblotted in FIGURE 15B4.

FIGURE 15B4 shows Western immunoblots measuring the level of expression of cyclin E in elutriated subpopulations of cells from FIGURE 15A by the methods described in FIGURE 15B2, but using anti-cyclin E and ^{125}I-Protein A to visualize the level of cyclin E instead of anti-CDK2.

FIGURE 15C shows graphically the level of ^{125}I-Protein A bound by anti-cyclin E:cyclin E bands in Western immunoblots as a function of the amount of cellular extract (elutriated fraction 3 extract) used in the method of FIGURES 15B1-B4. The results show that increasing the amount of cyclin E increased the amount of signal in the immunoassay for cyclin E.

FIGURE 16, described in Example 13, shows the results of studies designed to investigate the molecular association of cyclin E with CDC2 and CDK2; assembly of cyclin E:CDC2 or cyclin E:CDK2 complexes *in vitro*,;and activation of phosphorylase kinase activity following association of the kinases with cyclin E.

FIGURE 16A shows the results of experiments assaying phosphorylase kinase activity in cyclin E:CDK2 complexes immunoprecipitated with antibody specific for CDK2 (Anti-CDK2). The complexes were formed in the cell extracts of hydroxyurea-arrested cells (HU) and in extracts of G1 phase cells (G1 extract) in the absence (0) or presence of differing amounts (5,1,0.2) of recombinant cyclin E. Kinase activity in the immunoprecipitates was determined using histone H1 as a substrate, SDS-PAGE, and phosphor imaging of the ^{32}P-labeled histone H1 bands in the gels. The results show that the addition of cyclin E to G1 cell extracts activated latent kinase activity in the extracts in a dose-dependent manner, i.e., the level of kinase activity measured was dependent upon the amount of cyclin E added.

FIGURE 16B shows the results of experiments assaying phosphorylase kinase activity in cyclin E complexes. The experiments were conducted as described in FIGURE 16A, above, but using antibodies specific for cyclin E (Anti-cyclin E) instead of Anti-CDK2. The results confirm those presented in FIGURE 16A, above: namely, cyclin E activates a latent CDC kinase activity in extracts of G1 cells in a dose-dependent manner.

FIGURE 16C shows the results of experiments designed to assay for the phosphorylase kinase activity in cyclin E:CDC2 complexes. The experiments were conducted as described in FIGURE 16A, above, but using antibodies specific for CDC2 (Anti-CDC2). The results show minimal to no CDC2 kinase activity in G1 extracts of cells even when cyclin E was added.

FIGURE 17 graphically depicts the results obtained in FIGURES 16A, 16B, and 16C, above. The results obtained with each respective G1 cell extract immunoprecipitate (CDC2, solid/left-most bars in set of three bars; CDK2, hatched bars/middle of each set; or, cyclin E, shaded/right-most bars in each set of three) is expressed as a percentage of the phosphor imaging signal obtained with the kinase in the HU-cell extract immunoprecipitate (i.e., 100%; %hydroxyurea H1 kinase). The numbers at the top of each bar are the maximal value recorded in percent (%). The results show that cyclin E activated a latent CDK2 kinase activity in the G1 cell extracts and the activity of the kinase was dependent upon the amount of cyclin E added (i.e., expressed as the fold-dilution of the cyclin E added to the HU extract; "fold cyclin E in HU extract"). The results indicate that the availability of cyclin E is a factor controlling phosphorylase kinase activity during the G1 phase of the cell cycle.

FIGURE 18, described in Example 14, graphically represents the effects of serum growth factors on the rate at which LXSN-*cyclin E*-transduced Rat-1 cells and LXSN-transduced control cells initiate DNA synthesis following nocodazole-arrested

mitosis. The incorporation of BrdU into nuclear DNA was measured in LXSN-*cyclin E*-transduced (RAT1/cyclin E) or LXSN-transduced (RAT1/LX) control cells as a function of time after the nocodazole mitotic block. Only cells synthesizing DNA (i.e., S-phase cells) incorporate BrdU into DNA and scoring the number of nuclei in a cell culture (i.e., % labelled nuclei) can thus be used to evaluate the rate of transition of the cells from G1 into the S-phase.

FIGURE 18A show the results of experiments designed to evaluate the rate at which LXSN-*cyclin E*-transduced (RAT1/cyclin E) or LXSN-transduced (RAT1/LX) control cells initiate DNA synthesis following release of a nocodazole block. The results show that the *cyclin E*-transduced cells initiated DNA synthesis more than 2-3 hours earlier than control-transduced cells, and the rate of nuclear labeling (i.e., initiation of DNA synthesis) was also greater in the cyclin E-transduced cells (as evidenced by the differing slopes of the two curves).

FIGURE 18B shows the results of experiments designed to evaluate the growth factor dependence for initiating DNA synthesis in LXSN-*cyclin E*-transduced and LXSN-transduced control cells. Both types of cells were cultured in medium containing either 1% or 0.1% bovine calf serum. The results show that a) LXSN-*cyclin E*-transduced cells initiated DNA synthesis more rapidly than control cells following release of the nocodazole block in either 1% or 0.1% serum, and b) *cyclin E*-transduced cells were less dependent on growth factors than LXSN-transduced control cells as evidenced by their more rapid initiation of DNA synthesis in low serum (i.e., the LXSN-*cyclin E*-transduced cells initiated DNA synthesis more than 6-8 hours earlier than LXSN-transduced cells in 0.1% serum), and the cyclin E-transduced cells also proliferated more rapidly in the low serum conditions that the control (i.e., as determined by comparing the slopes of the the two transduced cell types grown in 0.1% serum).

Detailed Description of the Preferred Embodiment

A new human cyclin, named cyclin E, was isolated by complementation of a triple *cln* deletion in *S. cerevisae*. Cyclin E showed genetic interactions with the *CDC28* gene suggesting that it functioned at START by interacting with the CDC28 protein. Two human genes were identified that could interact with cyclin E to perform START in yeast containing a *cdc28* mutation. One was *cdc2-HS* and the second was the human homolog of *Xenopus CDK2*. Cyclin E produced in *E. coli*, bound and activated the CDC2 protein in extracts from human G1 cells, and antibodies against cyclin E immunoprecipitated a histone H1 kinase from HeLa cells.

The interactions between cyclin E and CDC2, or CDK2, may be important at the G1 to S transition in human cells.

The invention provides nucleic acid molecules capable of hybridizing under stringent conditions to the human cyclin E cDNA shown in FIGURE 2 from position 1 to 1185. Although only a single (+) strand of the cDNA is shown in FIGURE 2, those skilled in the art will recognize that its complementary (-) strand is thereby disclosed as well. By nucleic acid molecule is meant DNA, RNA, and/or synthetic nucleotide sequences such as oligonucleotides that are the same as, homologous with, or complementary to, at least one helical turn (about 10 to 15 nucleotides) of the illustrated *cyclin E* nucleotide sequence. The invention provides more than three *cyclin E* cDNAs resulting from alternative splicing of *cyclin E* mRNAs, genetic polymorphism, and translocation in tumorigenesis. Those skilled in the art will recognize that the members of this closely related group of *cyclin E* nucleic acids are readily identified by their ability to hybridize under stringent conditions with all or portions of the nucleotide sequence of FIGURE 2 or its complementary (-) strand. By capable of hybridizing under stringent conditions is meant annealing of a nucleic acid molecule to at least a region of the disclosed *cyclin E* nucleic acid sequence (whether as cDNA, mRNA, or genomic DNA) or to its complementary strand under standard conditions, e.g., high temperature and/or low salt content, which tend to disfavor hybridization of noncomplementary nucleotide sequences. A suitable protocol (involving 0.1 x SSC, 68°C for 2 hours) is described in Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, 1982, at pages 387-389. Such hybridizing nucleic acid molecules may be related to the disclosed sequence by deletion, point mutation, base substitution, frameshift, alternative ORFs, mRNA splicing and processing, or post-transcriptional modification (e.g., methylation and the like). For example, antisense nucleic acids are provided having nucleotide sequences complementary to the *cyclin E* sequence and characterized by the ability to inhibit expression of a *cyclin E* gene, e.g., by binding and inhibiting translation of a *cyclin E* mRNA. Antisense nucleic acids may be encoded within a host cell, e.g., following transduction or transfection of the cell with a vector DNA or RNA sequence encoding an antisense nucleic acid, or, alternatively, the antisense nucleic acids may be synthetic oligonucleotides. Such antisense oligonucleotides are introduced into cells by a variety of means, e.g., with retroviral vectors encoding antisense mRNA in the cell, or by fusing the cell with liposomes containing an antisense oligonucleotide and the like. The subject antisense nucleic acid molecules are characterized by their

ability to hybridize under stringent conditions with the illustrated cyclin E nucleic acid, its complementary strand, 5' transcription regulatory regions of a *cyclin E* gene, or translation regulatory regions of a *cyclin E* mRNA.

The isolated nucleic acids of the invention preferably encode cyclin E polypeptides. Such polypeptides are not necessarily encoded by the aforesaid isolated nucleic acid molecules, since those skilled in the art will recognize that the disclosed *cyclin E* nucleotide sequence permits construction of a variety of synthetic polypeptides. Such synthetic polypeptides may vary in length (e.g., from about 5 amino acids to many hundreds of amino acids) and be constructed corresponding to selected regions of the encoded cyclin E polypeptide. The subject cyclin E polypeptides thus encompass isolated cyclin E polypeptides (i.e., found in normal cells), mutant polypeptides (e.g., resulting from mutagenesis, or found in tumor cells), and chemically modified polypeptides (e.g., having one or more chemically altered amino acids, in which case a designated amino acid can be converted into another amino acid, or chemically substituted or derivatized and the like). Functional sites in the cyclin E polypeptides are identified by constructing mutants of the cyclin E nucleic acid, e.g., and testing the constructs for expression products having altered functional properties such as failure to bind or activate a CDC protein kinase, or failure to advance the cell cycle. Particularly useful for constructing such mutants are regions of conserved nucleotide or amino acid sequence, e.g., conserved between cyclins A, B, C, D, and E, or conserved among the members of the cyclin E family. Conserved regions of cyclin E are functional and protein-structural regions of the polypeptide.

In an illustrative preferred aspect, expression of a cyclin E polypeptide in a cell allows levels of cyclin E in the cell to rise to a point where cyclin E binds and activates a CDC protein kinase, and eliminates certain growth factor and serum requirements for progression of the cell through the G1 phase of the cell cycle. As a result, the G1 phase of the cell cycle is shortened. The G1 phase commonly lasts about 8 to about 12 hours, and expression of a cyclin E polypeptide in a cell (or exposure of a cell to a cyclin E polypeptide) may shortened G1 phase by about 1 hour to many hours. That a cyclin of the invention shortens the G1 phase of the cell cycle can be readily determined by those skilled in the art by using a model test system such as that provided below in the Examples, e.g., by the "598-5" or "1238-14C" strains of yeast. Alternatively, a mammalian cell such as an NIH3T3 cell may be transfected or transduced with an expression vector containing a cyclin E-hybridizing nucleic acid and the length of the G1 phase of the 3T3 cell cycle can then be determined in kinetic cell cycle assays such as those illustrative examples provided below. For instance,

those skilled in the art will recognize that progression of cells from M to S phase can be measured (i.e., in hours and minutes) by determining tritiated thymidine or bromodeoxyuridine (BrdU) incorporation. Cyclin E nucleic acid, when transfected or transduced into test cells, induces either a faster progression of the cells from the M phase to the S phase; or a progression of the cells from M to S without requiring exogenous stimulae, i.e., serum or growth factors and the like. In either case, introducing the subject cyclin into the test cell results in a shortening of the G1 phase and a more rapid progression from M to S.

Representative examples of CDC protein kinases to which cyclin E binds include CDC2, CDC28, CDCK2-XL, CDC2-HS, and CDK2-HS. (Note that in this terminology "HS" designates *Homo sapiens* and "XL", *Xenopus laevis*.) The invention also provides methods for identifying and cloning other CDC kinases that are bound and activated by cyclin E (see Example 10, below). Those skilled in the art will understand that synthetic cyclin E polypeptides may be readily constructed by modifying the disclosed amino acid sequence and testing for altered functional properties, i.e., altered binding, activation of a CDC protein kinase, and/or altered ability to shorten the G1 phase of the cell cycle. Such synthetic cyclin E polypeptides are useful competitive and noncompetitive inhibitors of a normal or mutant cyclin E (i.e., derived from a normal or mutant cell) or of its CDC protein kinase binding partner. Such synthetic polypeptides also include polypeptide antagonists or agonists useful for changing the functional properties of the cyclin E:CDC protein kinase complex, e.g., by increasing, decreasing, or otherwise modifying or modulating: a) the phosphorylase activity activated by the CDC protein kinase; b) the activity of the cyclin E, e.g., for activating the CDC protein kinase; c) the cell cycle promoting activity of the cyclin E:cell division kinase complex; and/or, d) transcriptional regulatory factors that bind the 5' region of the *cyclin E* gene.

Skilled artisans will further understand that the disclosure herein of recombinant *cyclin E* nucleic acids, cells, and *in vitro* assays provide opportunities to screen for compounds that modulate, or completely alter, the functional activity of a cyclin E protein or *cyclin E* nucleic acid in a cell. In this context "modulate" is intended to mean that the subject compound increases or decreases one or more functional activity of a cyclin E protein or nucleic acid, while "alter" is intended to mean that the subject compound completely changes the cyclin E protein or nucleic acid functional activity to a different functional activity. In this context, an example of a compound that "modulates" the activity of a cyclin E protein is an inhibitor capable of decreasing the level of CDC kinase activity following binding of a cyclin E

to the CDC kinase; and, an example of a compound that "alters" the activity of a cyclin E protein is an agent that induces cyclin E to bind to CDC2 instead of to CDK2.

The screening assays illustrated in the Examples (below) include biochemical assays (e.g., measuring effects of cyclin E protein on CDC2 and CDK2 phosphorylase activity), and cellular *in vitro* assays (e.g., measuring the effects of *cyclin E* expression on cell proliferation). The illustrative biochemical assays may be particularly useful in screening for compounds modulating a cyclin E molecular activity, while the cellular assays may be particularly useful in screening for compounds altering a cyclin E activity in a cell. For example, in proliferating cells cyclin E participates with other cyclins, CDC kinases, growth factor second messengers, transcription regulatory factors and the like in controlling the proliferative response of a cell to its environment. Those skilled in the art will understand that binding of a ligand at a molecular binding site can be modulated in a direct manner (e.g., by blocking the site), as well as altered in an indirect manner (e.g., by conformational changes induced following binding of a second (different) ligand at a distant site). In this regard, it is likely that the binding site specificity of cyclin E for a particular CDC kinase (or some other cellular control factor, as discussed below), can be completely altered (i.e., to bind a different ligand) by agents that bind at distant sites in the cyclin E polypeptide. Examples of compounds that may be screened in the latter several assays include at least nucleic acids (e.g., DNA oligonucleotide aptamers that bind proteins and alter their functions), proteins, carbohydrates, lectins, organic chemicals, and the like. Such screening assays may be useful for identifying candidate therapeutic agents that may provide drugs useful in animals and humans.

It is still further understood that, due to the significance of cyclin E and the cyclin E:CDC protein kinase in the cell cycle, innate regulatory mechanisms exist in cells for regulating their activity by binding to cyclin E or to complexes containing cyclin E. Such regulatory factors can include, at least: a) cofactors that bind to the complex and exert regulatory action by destabilizing or stabilizing the complex; b) agents that modulate or alter the activity of the complex by inducing conformational changes in the CDC protein kinase and/or cyclin E polypeptides as they are bound together in the complex; c) enzymes that inactivate one or both members of the complex; and, d) cellular control factors (e.g., signal transduction second messengers, transcription regulatory factors, and the like) that bind cyclin E or cyclin E complexes and modulate or alter functional activity. Thus, artificial

polypeptides can be constructed that control the activity of the cyclin E:CDC protein kinase kinase complexes in the cell by inhibiting or promoting the activities of such regulatory factors. Those skilled in the art will recognize that the functional regions of cyclin E represent particularly attractive targets for three-dimensional molecular modeling and for the construction of mimetic compounds, e.g., organic chemicals constructed to mimic the three-dimensional interactions between the cyclin E and its CDC protein kinase binding partner. In a particularly preferred embodiment, the invention provides isolated nucleic acid molecules that encode artificial cyclin E polypeptides that bind to, but do not activate, CDC protein kinases.

In other preferred embodiments of the invention, polypeptides are provided that are encoded by nucleic acids corresponding to the following regions of the cyclin E nucleotide sequence (i.e., regions that are conserved between cyclins A, B, and E): namely, a) a carboxy-terminal leucine repeat sequence (i.e., residing between positions 640 and 1185, and more particularly between positions 631 and 936, of the cyclin E cDNA shown in FIGURE 2); b) an MRAIL sequence (i.e., residing between positions 385 and 645 of the cyclin E cDNA shown in FIGURE 2); and c) a C-terminal sequence region (i.e., residing between positions 1048 and 1080 of the cyclin E cDNA shown in FIGURE 2). The MRAIL sequence is necessary, but not sufficient, for binding to a cell division kinase.

It is further understood that mutant *cyclin E* nucleotide sequences may be constructed from the sequence shown in FIGURE 2. The subject mutant cyclin E nucleotide sequences are recognized by their ability to encode mutant cyclin E polypeptides that may have binding affinity for a CDC kinase (e.g., CDK2) polypeptide that is higher or lower than that exhibited by a cyclin E polypeptide for a CDC kinase in a non-transformed mammalian cell. The subject mutant polypeptides also may alter (i.e., increase or decrease) the enzyme activity of the CDK2 kinase when the subject mutant cyclin E polypeptide and CDK2 are resident together in a complex. Illustrative examples of changed enzyme activity include: a) increased or decreased enzymatic activity (e.g., Km, Vmax, kcat, and the like); b) changed stability of the kinase in the complex (e.g., to time-dependent decay of the complex or the enzyme activity); c) changed susceptibility of the kinase to proteolytic inactivation; d) changed susceptibility of CDK2 to dissociate from the cyclinE:CDK2 complex in response to binding of regulatory factors (discussed above) by the complex; or e) changed sensitivity of the kinase in the complex to competitive or noncompetitive inhibitors. Skilled artisans will recognize a variety of methods by which the sequence in FIGURE 2 may be mutated (e.g. with chemical agents or radiation), and by which

clones of cells containing the mutated *cyclin E* nucleotide sequences may be identified and/or selected. The subject mutant *cyclin E* nucleotide sequences are useful for modulating or altering the activity of a cyclinE:CDK2 complex in a cell, e.g., in a tumor cell to decrease CDK2 kinase activity and slow cell proliferation, or in a terminally differentiated cell to increase CDK2 kinase and stimulate growth. The subject mutant *cyclin E* nucleotide sequences may be introduced using vectors such as the illustrative retroviral vectors in Example 9.

Artificial cyclin E polypeptides, organic chemical mimetics, antisense RNA and oligonucleotides, and the like find broad utility as selective inhibitors of cell proliferation triggered by growth factors, mitogens, cytokines, and like agents, without inhibiting ongoing reparative mitotic activity in a tissue. Thus, it will be appreciated that the synthetic polypeptides, mimetics, and antisense embodiments of the invention will preferably exhibit differential inhibitory activities; e.g., when the subject inhibitor is introduced into two cells, one triggered by a cytokine to proliferate, and a second undergoing mitosis, the first cell is inhibited but the second cell is not. The subject synthetic cyclin E polypeptides of the invention only inhibit cells that are transitioning from G1 to S, and not those cells that are already actively undergoing mitosis. Thus, those skilled in the art will recognize that representative examples of utility include inhibiting induction of immune responses; interrupting clonal expansion (i.e., either T or B lymphocyte) of an ongoing immune response; inhibiting growth factor-induced proliferation of tumor cells or metastatic cells that are transitioning from G1 to S; and inhibiting growth factor-induced tissue hypertrophy (e.g., vascular smooth muscle cell proliferation such as in atherosclerotic plaques, mesenchymal hypertrophy of fibroblasts and connective tissue cells such as in rheumatic joints, and the like). The subject selective inhibitors are conveniently recognized, for instance, by their ability: a) to either decrease (or increase) the levels of cyclin E polypeptide or mRNA in a test cell *in vitro* (i.e., compared to a control cell of the same type); b) to decrease (or increase) the level of cyclin E that can form a complex with CDK2; or c) to decrease (or increase) the binding affinity of cyclin E polypeptides for members of the CDK2 family of cell cycle dependent kinases in mammalian cells. Skilled artisans will recognize that measuring a decreased (or increased) activity of a subject selective inhibitor may be accomplished using asynchronized or synchronized cell cultures; e.g., in synchronized cultures of cells cyclin E levels and activities are examined during the G1 phase of the cell cycle.

Aspects of the invention include recombinant expression vectors such as viral vectors for mammalian cells (e.g., retroviruses similar to that illustrated in Example 9,

vaccinia virus, adenoviruses, CMV, and the like), and plasmid or cosmid vectors useful for transfecting and transducing nucleic acid into prokaryotic and eukaryotic cells. Recombinant expression vectors of the invention are constructed for example by operably linking a *cyclin E* nucleic acid to suitable control sequences. Operably linking is used herein to mean ligating a *cyclin E* nucleic acid to an expression vector nucleic acid in a manner suitable for transcription and translation of *cyclin E*, preferably under a predetermined positive (or negative) regulatory control exerted by control sequences in the expression vector (i.e., containing regulatory sequences capable of driving expression, over-expression, and constitutive-expression of the cyclin E gene, e.g., promoter, enhancer, operator sequences, and the like). Selectable markers will generally be included in the expression vector. Representative examples of such selectable markers include enzymes, antigens, drug resistance markers, or markers satisfying the growth requirements of the cell. It will also be appreciated that in certain cells transfection or transduction with *cyclin E* nucleic acid will provide a selective proliferative/growth advantage that will serve as a type of selectable marker (e.g., in mutants blocked for progression of the cell cycle, such as the representative yeast strains "598-5" or "1238-14C" described below in the Examples). The subject expression vectors are useful for transfecting and transducing cells to produce cyclin E polypeptides, mutant cyclin E polypeptides, and antisense nucleic acids. For instance, aspects of the invention include methods for using the transfected and transduced cells to produce polypeptides that are able to activate a CDC protein kinase and advance the cell cycle at the restriction point from the G1 phase to the S phase. Several methods are available for determining that a polypeptide encoded by a *cyclin E* nucleic acid is capable of advancing the cell cycle. Representative examples involving yeast cells and mammalian cells are described in the Examples, below.

The invention also provides a cell type that has been transduced or transfected with a cyclin (e.g., cyclin E) expression vector. In one preferred embodiment a cell constitutively over-expressing *cyclin E* proliferates at a rate faster than its parent cell. Cyclin E-transduced human diploid fibroblasts illustrated in Example 14, below, were 20-50% smaller in length and width than control-transduced or normal cells. Skilled artisans will understand the advantages in gene therapy of small rapidly growing cells, e.g., cells that may be grown in a cost-efficient manner and that may undergo programmed senescence faster than their normal counterparts. It will also be understood that transgenic animals (e.g., experimental and domestic animals) may be constructed of such small rapidly growing cells. Skilled artisans will recognize that the advantages offered by the subject cells include: a) improved growth in tissue

culture of terminally differentiated cell types that would normally be difficult or impossible to culture (e.g., stem cells); and b) lessened (or no) dependence on growth factors for cell growth (e.g., for cells that are difficult to propagate *in vitro* such as muscle or neural cells), allowing more rapid growth of cultures of mammalian cells in low-serum (or serum-free) medium (e.g., in production cultures of cells manufacturing biotherapeutic agents). The disclosure herein identifies the singular significance of a cyclin in determining the progression of the cell cycle at each of several decision points. The term "decision point" is well accepted in the art as meaning a point in the cell cycle where a cell may arrest its growth until such time as an appropriate signal is received to progress the cell cycle. The results presented in Example 14, below, illustrate the significance of cyclin E in activating a latent CDK2 kinase activity at a decision point in the G1phase of the cell cycle. Several decision points exist during the different phases of the cell cycle the disclosure herein indicates that a small number of cyclins may be operative in the cell cycle, i.e., one cyclin to advance the cell cycle at each decision point. Thus, over-expression of a relatively small number of cyclins in a cell may be sufficient to render the cell nearly completely independent of exogenous growth factors.

In other embodiments the invention provides immunologic binding partners for cyclin E polypeptides such as polyclonal and monoclonal antibody molecules, and various antigen-binding fragments thereof, capable of specifically binding the cyclin E polypeptide. Such immunologic binding partners may be produced by hybridoma or rDNA expression techniques and find utility in therapeutic, purification, and diagnostic applications. Therapeutic applications include binding partners that inhibit binding of a cyclin E to its CDC protein kinase; binding partners that modulate CDC protein kinase; and binding partners that alter regulatory control of *cyclin E* in a cell. Representative examples of purification applications include immunochemical methods and immunoaffinity chromatography. Representative examples of diagnostic applications include enzyme-linked and radioisotopic immunoassays, immunofluorescence, fluorescence immunoassay, time-resolved fluorescence immunoassay and the like. The specific binding partners used in these assays may be capable of distinguishing between free cyclin E polypeptide and cyclin E bound in complex with CDC protein kinases. Those skilled in the art will recognize that "neo" (new) antigens are acquired by conformationally altered polypeptides, and will further recognize that the binding between cyclin E and a CDC protein kinase induces such a conformational alteration in both polypeptides. The cyclin E-specific binding partners find general utility in diagnostic assays for detecting and quantitating levels (e.g.,

protein or antigen) and functional activities (e.g., phosphorylase kinase activation) of free cyclin E (or complex-associated cyclin E) in a cell such as a tumor cell. The subject diagnostic assays include assays for: a) detecting the absolute levels and activities of cyclin E in nonsynchronized cell populations (e.g., in tumor biopsy specimens); b) comparing the levels and activities of cyclin E in synchronized cell populations at different phases of the cell cycle (e.g., cell populations synchronized by thymidine-block); and c) assays for determining the levels and activities of cyclin E in biological fluids (i.e., blood, serum, plasma, mucus secretions, CNS fluid, cell extracts, and the like). The absolute levels and activities of cyclin E expressed in malignant biological fluids (e.g., tumor cell extracts, serum from cancer patients, and the like), as well as the levels and activities expressed in cell extracts prepared at different stages in the cell cycle, may provide information on the rate of cell growth. In this regard the assayed levels and activities of cyclin E may serve as diagnostic markers for:

a) staging tumors, since differentiated cells grow more slowly that transformed; express lower levels of cyclin E protein and activity than transformed cells; and (in contrast to transformed) differentiated cells express cyclin E only during G1 phase of the cell cycle;

b) determining prognosis, i.e., predicting patient survivability and time to recurrence of tumor, because rapidly growing malignant cells capable of metastasis may generally grow more rapidly than differentiated cells; exponentially growing cells may express higher absolute levels (or activities) of cyclin E, or alternatively, the higher levels (or activities) of cyclin E may be present during a particular phase of the cell cycle, e.g., during S, G1 or G2 phase; and/or

c) predicting therapeutic success, i.e., of a particular therapeutic regimen, because more slowly growing cells may express lower levels (or activities) of cyclin E (i.e., than rapidly growing metastatic cells) and also be more responsive to less drastic and more prolonged therapeutic regimens.

The subject assays for determining the level (or activities) of cyclin E in cells may also indicate the responsiveness of a patient's tumor to a particular therapeutic agent exerting its affect on cells during the G1 phase of the cell cycle. Those skilled in the art will recognize that the subject diagnostic assays may provide results that are potentially useful to a physician in deciding how to stage a tumor, how to select an appropriate therapeutic regimen, how to evaluate the success of therapy, and how to evaluate patient risk or survivability.

In other embodiments, the invention provides diagnostic assays for measuring the absolute levels (or functional activities) of cyclin E:CDK 2 polypeptide complexes in asynchronous cells, and the levels (or activities) of the cyclin E:CDK 2 complexes at different stages in the cell cycle. In nontransformed cells the peak abundance of the cyclin E:CDK 2 complexes occurs late in the G1 phase of the cell cycle, e.g., with levels 4-fold to 6-fold higher than in other phases of the cell cycle. In transformed cells constitutive over-expression of cyclin E and/or CDK 2 may lead to differences in the levels of the cyclin E:CDK 2 complexes that are detectably different during the G1, G2, M, and S phase of the cell cycle. The subject assays that determine the levels of cyclin E/CDK 2 complexes in cells may be useful in assessing malignant cells from patients, e.g., as described above.

In other embodiments the invention provides diagnostic assays for determining chromosomal rearrangement of *cyclin E* and *CDK2* genes in a cell. The chromosomal location of *cyclin E* and *CDK2* genes is conveniently determined in chromosomal smears by *in situ* hybridization with oligonucleotide probes or cDNA and the like. Translocation of a *cyclin E* gene or *CDK2* gene, i.e., from a chromosomal location found in a normal cell to a location found in a transformed cell, may contribute to a phenotype of uncontrolled cell growth by removing normal transcription regulatory control of either gene expression of a cyclin, e.g., *cyclin E,* or a CDC kinase, e.g., *CDK2*. The findings disclosed herein indicate heretofor unrecognized common junction points where second messenger signals from multiple growth factors converge at a small number of different cyclin:CDC kinase complexes. The outcome of the molecular interaction of the second messengers with the cyclin:CDC kinase complexes determines whether the cell progresses the cell cycle. Thus, rearrangement of a cyclin gene in a cell may have dramatic results. In the case where the rearrangement induces over-expression the cell may acquire a malignant (i.e., uncontrolled) growth phenotype, and in the case where the rearrangement induces under-expression the cell may undergo premature senescence. Screening cellular samples from individuals for the potential of *cyclin E* or *CDK2* chromosomal rearrangement may indicate a relative risk factor for the possibility of developing cancer. In the event that such rearrangements are detected, restoring normal control of a cyclin gene (e.g. *cyclin E)* or a CDC kinase gene (e.g. *CDK2*) in a translocated chromosomal location may reverse the malignant phenotype of a transformed cell. For example, the cyclin E gene (of CDK2 gene) and its regulatory elements may serve as a targets for gene therapy vectors designed to inactivate the rearranged gene, e.g.,

using *in situ*-directed recombination/mutagenesis or targeted integration to disrupt the translocated gene.

The invention also provides transgenic strains of yeast cells that are engineered to contain a genome lacking in *cln1*, *cln2*, and *cln3* genes required for cell cycle progression but having an episomal nucleic acid capable of encoding CLN3. A representative embodiment is yeast strain "589-5", which is useful to identify mammalian cyclin genes. Strain "589-5" has been deposited with the American Type Culture Collection, Rockville, MD, under accession No. 74098. When *cyclin E* nucleic acid is introduced into the transgenic yeast cells of this strain, the cells advance through the cell cycle from START into S phase. While others have shown previously that somewhat similar transgenic yeast constructions are useful for identifying and cloning yeast *cdc* genes, the subject strains are useful for identifying and cloning mammalian *cyclin E* cDNA, and also for identifying and cloning mammalian *cdc* protein kinase cDNAs.

The invention also provides other transgenic strains of yeast cell, having a *cdc28-13* gene, an endogenous G1 *CLN* (e.g., *CLN3*) under control of a conditional promoter (e.g., *GAL*), a mitotic CLB cyclin (e.g., *CBC*), and a *cyclin E* gene, such as the representative yeast strain "1238-14C-*cyclin E*". In this case the conditional promoter drives expression of the G1 cyclin in the presence of a factor ("the condition") required for metabolism or growth. The G1 cyclin, in turn, binds and activates the cell division kinase encoded by the *cdc28-13* gene, and activation of the CDK allows the cells to be grown and passaged. Strain "1238-14C-*cyclin E*" has been deposited with the American Type Culture Collection, Rockville, MD, under accession No. 74099. The cell cycle is blocked in these cells until a *cdc* protein kinase nucleic acid is introduced. Thus, this strain of transgenic cells is useful for identifying and cloning *cdc* protein kinases that associate with a cyclin E and advance the cell cycle from G1 into S phase in a eukaryotic host cell.

The invention also provides methods for cloning regulatory agents such as polypeptides that bind to cyclin E:CDC protein kinase complexes and inhibit or promote the activity of the complex, or that change the half-life of the complex, and the like. Nucleic acids encoding such regulatory agents are identified by introducing a candidate nucleic acid molecule into a transgenic strain of yeast cell, or a mammalian cell, in which advance of the cell from G1 into S phase is dependent upon the activity of a cyclin E:CDC protein kinase. A representative example of a transgenic yeast strain useful in this manner is provided by strain 598-5 transfected or transduced with a *cyclin E* gene, such as strain HU4 described in greater detail in the Examples below.

Nucleic acids encoding regulatory agents are recognized by their ability to inhibit progression of the cell from G1 into S. Thus, in the case of the described transgenic yeast strain, replicative screening techniques may be used for identifying clones of cells that fail to advance the cell cycle at START after they have been transfected or transduced with any candidate cDNA, e.g., mammalian, insect, avian, reptilian, amphibian, etc.

EXAMPLES

The data set forth below show that cyclin E substituted for the *S. cerevisae CLN* genes and interacted with CDC28 to perform START. At least two different members of the human *CDC2* gene family could interact with cyclin E to regulate START in budding yeast, *CDC2-HS* and *CDK2-HS*. We have also shown that the cyclin E protein bound and activated the p34 CDC2 protein in extracts from human lymphoid G1 cells and that cyclin E was associated with an H1 kinase activity in HeLa cells. Others found that the cyclin E mRNA was specifically present during the late G1 phase of the HeLa cell cycle (Lew et al., 1991). Taken together these results argue that cyclin E may function as a regulator of the p34 CDC2 kinase at the G1 to S transition in the human cell cycle.

The interpretation of our result that cyclin E rescued the triple *cln* deletion was complicated by the fact that human cyclin B, which is a mitotic cyclin, also substituted for the yeast *CLN* genes. Regardless of the mechanism by which cyclin B functioned as a CLN protein, the fact that it played this role implied that our complementation assay did not specifically identify CLN-type cyclins. Therefore, a more complete understanding of the function of cyclin E must await analyses of the abundance of cyclin E protein and its association with CDC2, or CDC2-related proteins, during the cell cycle of normal human cells.

In yeast, and probably in most organisms, the CDC2 protein functions at least twice during the cell cycle: at G1/S and again at G2/M. At each point the CDC2 protein associates with a unique type of cyclin: the B-type cyclins at G2/M and the CLNs (at least in budding yeast) at G1/S. Therefore, it had been expected that the unique cyclin-CDC2 complex which assembled at each control point would impart the specificity required for the CDC2 kinase to activate either S phase or mitotic events. For example, the substrate specificity or subcellular localization of the CDC2 kinase would be determined by its particular cyclin partner. The ability of human cyclin B to substitute for the CLN proteins appears, on the surface, to contradict this simple hypothesis. As an alternative it is possible that the specificity of p34 CDC2 action at different points in the cell cycle might be determined, at least in part, by the CDC2

protein itself. This could be due to cell cycle-specific modification of the CDC2 protein (Simanis & Nurse, 1986; Lee et al., 1988; Gould & Nurse, 1989; Krek & Nigg, 1991) or, in higher eukaryotes, to the activation of different members of the *CDC2* gene family at different points in the cell cycle (Paris et al., 1991; Pines & Hunter, 1990). Our observation that at least two different members of the *CDC2* gene family can perform START in yeast is consistent with this idea. In this model periodic accumulation and destruction of the cyclin proteins would determine the timing of p34 kinase activation but not its specificity. A model similar to this has been proposed previously based upon the phenotypes of certain *cdc2* mutants in *S. pombe* (Broek et al., 1991). An alternative is that the particular substrates necessary for CDC2 or CDC28 to induce the S or M state become accessible in a cell cycle-dependent manner. We point out, however, that the design of our experiments may not have permitted all the normal controls on cyclin specificity to be observed. For example, expression of human cyclin B from the strong *ADH* promoter might have overwhelmed certain regulatory processes that usually limit cyclin B-CDC28 activity to the G2/M transition.

Human cyclin E is more closely related to human cyclins A and B than to the budding yeast CLN proteins. Within the cyclin box the level of identity to CLN1 is 21% and to CLN3, 17%. This compares to 49% identity to human cyclin A and 44% to human cyclin B. Outside the cyclin box region cyclin E shows no extensive homology to either the human cyclins or yeast CLN proteins. On this basis cyclin E does not appear to be a direct homolog of the yeast CLN genes. This comparison must be made with caution, however, since the precise regions within the various cyclins that determine their functional differences have not been identified. In addition, the similarity among the human cyclins may reflect, to some extent, their co-evolution with common targets such as the human CDC2 protein.

Two other features of the cyclin E sequence should be noted. Our clone of cyclin E lacks an N-terminal sequence found in both cyclin A and B, which is thought to be a recognition motif for the ubiquitination enzyme that mediates their destruction in mitosis (Glotzer et al., 1991). Furthermore, cyclin E contains a C-terminal extension when compared to cyclins A and B. This C-terminal region is flanked by basic residues and is rich in P (Pro), E (Glu), S (Ser), and T (Thr) residues. Such "PEST" regions have been implicated in controlling protein turnover (Rogers et al., 1986). In fact, the stability of the yeast CLN proteins may be determined by their C-terminal domains which are also rich in P, E, S, and T residues (Nash et al., 1988;

Cross, 1990; Hadwiger et al., 1989). These observations suggest that the stability of cyclin E during the cell cycle might be controlled differently than the mitotic cyclins.

In higher eukaryotes the role of the CDC2 protein during progression through the G1 phase of the cell cycle is not well understood. A number of independent observations suggest that the CDC2 protein has an essential function during this part of the cell cycle. Depletion of the CDC2 protein *in vivo* in human cells, using antisense oligonucleotides (Furakawa et al., 1990), or *in vitro* in *Xenopus* extracts, by immunoprecipitation (Blow & Nurse, 1990), can block the start of DNA synthesis. Addition of cyclin-CDC2 complexes from human S phase cells to extracts from G1 cells can activate DNA synthesis (D'Urso et al., 1990). However, a thermolabile mutation of the murine *CDC2* gene blocks entry into mitosis but not S phase at the nonpermissive temperature (Th'ng et al., 1990). Also, microinjection of antibodies against the yeast *CDC2* protein into human cells blocks the G2/M but not the G1/S transition (Riabowol et al., 1989). Our observation that at least two different members of the *CDC2* gene family can regulate the G1/S transition in *S. cerevisae* may help clarify these apparently contradictory results. One of these, the human *CDK2* gene, might preferentially work at G1/S as opposed to G2/M. Therefore, in higher eukaryotes, in contrast to the situation in yeast, multiple members of the *CDC2* gene family may participate in G1/S regulation. Under some circumstances their roles might be redundant, while in other situations they may all be essential.

Activation of the *CDC2* kinase during the G1 to S interval is likely to require its association with a cyclin. In *S. cerevisae* accumulation of a CLN-type cyclin is the rate-limiting step for transit through START (Nash et al., 1988; Cross, 1988; Hadwiger et al., 1989). In human cells activation of the p34 kinase at the start of S phase correlates with its assembly into a higher molecular weight complex (D'Urso et al., 1990; Marraccino et al., unpublished data), implying that association of p34 with a cyclin protein regulates its activity during this part of the cell cycle. We have also found that addition of purified recombinant clam cyclin A to a human G1 cell extract was sufficient to activate SV40 DNA replication, suggesting that accumulation of a cyclin is the limiting step for activation of the p34 kinase at the start of S phase.

At least four different human cyclins have been suggested to play roles during the G1 or S phases of the cell cycle. These include cyclins A (Giordano et al., 1989; Wang et al., 1990), C (Lew et al., 1991), D (or prad1; Motokura et al., 1991; Xiong et al., 1991; Matsushimi et al., 1991), and E (this disclosure; Lew et al., 1991). In yeast, human cyclin E can associate with either CDC2-HS or CDK2-HS to perform

START. *In vitro* cyclin E can bind and activate CDC2-HS, but its ability to activate CDK2 from G1 cells has not yet been tested. Human cyclin A has also been found to associate with two different members of the CDC2 gene family, CDC2-HS and a 33 kDa protein which may be CDK2 (Giordano et al., 1989; Pines & Hunter, 1990; R. Marraccino & J.R., unpublished observations). In contrast, the mitotic cyclin B has been found in association only with p34 CDC2 (see Hunt, 1989).

Multiple cyclins and CDC2-like proteins may be required to convey the diverse array of intracellular and extracellular signals that contribute to G1 regulation. Different members of the CDC2 protein family may preferentially interact with particular cyclins (Pines & Hunter, 1990). Also, each cyclin-CDC2 complex may perform only a subset of the events necessary for START to occur. Finally, it is possible that the CDC2 family of proteins may function at more than one point during G1. The START decision in yeast is the only clearly defined execution point for CDC28 during the G1 phase of the cell cycle. START bears certain similarities to the restriction point in the cell cycle of higher eukaryotes; however, the restriction point can occur hours before the start of S phase. Our *in vitro* replication experiments indicate that the CDC2 kinase may directly activate DNA synthesis (D'Urso et al., 1990). Therefore, CDC2, or related proteins, may function twice during G1, first at the point of commitment to cell proliferation and again at the onset of DNA synthesis. Each control point may require a unique set of cyclin proteins; for example the CLN-type cyclins may function at the restriction point and other cyclins, such as cyclin E, could act at the G1/S transition.

EXAMPLE 1

<u>Complementation of a yeast strain lacking CLN1, -2 and -3 with a human cDNA library</u>

Our initial goal was to identify human cDNAs encoding proteins that could substitute for the yeast CLN proteins. A yeast strain, 589-5, was constructed in which all three chromosomal *CLN* genes were deleted and which contained the *CLN3* gene under the control of the *GAL1* promoter on an episome. As CLN protein is required for passage through START, this strain will grow on galactose, where the *GAL1* promoter is induced; when this strain is grown on glucose the *GAL1* promoter is repressed, no CLN3 protein is made, and the cells arrest at START (Cross, 1990). A cDNA library (a gift of J. Colicelli and M. Wigler), using mRNA prepared from the human glioblastoma cell line U118, was constructed in a *S. cerevisae* vector containing the constitutive yeast *ADH* promoter for expression of the human cDNAs (Colicelli et al., 1989). The library was transfected into strain 589-5, and 10^5

independent transformants were screened, by replica plating, for their ability to grow on glucose. One transformant, HU4, was isolated whose growth on glucose was dependent upon expression of a human cDNA (FIGURE 1). For further experimental details, see the appended Materials and Methods.

EXAMPLE 2

HU4 encodes a new member of the cyclin protein family

The DNA sequence of the 1.7 kb HU4 cDNA is shown in FIGURE 2, and its homology, at the protein level, to human cyclins A and B is illustrated in FIGURE 3. The DNA sequence predicts a protein with 395 amino acids and a molecular weight of 45,120 daltons. *In vitro* transcription/translation of the HU4 cDNA yields a protein with the predicted molecular weight (see FIGURE 8). All known cyclins have a highly conserved central domain of approximately 87 amino acids. HU4 is 49% identical to human cyclin A and 44% identical to human cyclin B within this domain. On this basis we placed the HU4 protein within the cyclin family. N-terminal to this conserved region the homology to cyclin A falls to 5% identity, and 4% identity to cyclin B. C-terminal to this domain the identity to cyclin A is 14%, and the identity to cyclin B is 10%. This low level of homology both N- and C-terminal to the central conserved domain suggests that HU4 represents a new class of cyclin proteins, and we have designated this class as cyclin E. We cannot be certain that our cyclin E cDNA clone contains the entire cyclin E protein coding sequence, as the open reading frame extends to the 5' end of the sequenced cDNA. However, a cyclin E cDNA clone obtained from a MANCA cell library showed a 5' end identical to the one described here.

EXAMPLE 3

Human cyclin B complements the triple *cln* deletion

We compared the ability of human *cyclin A*, *B*, and *E* cDNAs to complement the triple *cln* deletion in strain 589-5. Full-length cDNA clones encoding human cyclins A (Pines & Hunter, 1990) and B1 (Pines & Hunter, 1989) were cloned into the ADH expression vector used in construction of the library described above. The various *cyclin E* expression plasmids were transfected into yeast by selecting for leucine prototrophy. The transformants were picked, and the ability of the human cyclin to complement the absence of the CLN proteins tested by growth on glucose. No leucine prototrophs were obtained using the *cyclin A* vector, suggesting that expression of full-length human cyclin A in *S. cerevisae* was lethal. FIGURE 4 depicts the relative plating efficiency of 589-5 on glucose versus galactose when containing either *cyclin B* or *E* expression plasmids. Surprisingly the mitotic *cyclin B*

complemented the absence of the CLN proteins. This experiment demonstrated that complementation of CLN function was not restricted to CLN type cyclins.

EXAMPLE 4

Interaction between cyclin E and *CDC28*

We compared the ability of cyclin E to rescue the triple *cln* deletion in isogenic strains containing either the *CDC28* or the *cdc28-13* allele at the permissive temperature of 30°C. FIGURE 4 shows that cyclin E substituted for the CLN proteins significantly less well in the *cdc28-13* background. This genetic interaction between the *cyclin E* and *CDC28* genes suggested that the cyclin E protein performed its function by interacting with the CDC28 protein. Cyclin B was about as effective in *cdc28-13* versus *CDC28* strains, suggesting that cyclin B might interact with CDC28 differently than cyclin E.

EXAMPLE 5

Human cyclin E and human *CDC2* can perform START in *S. cerevisae*

Strains containing *cdc28-13* and the endogenous G1 (CLN) and mitotic (CLB) cyclins are viable at 30°C. Therefore, the cdc28-13 protein must be capable of functional interactions with both types of cyclins. As described above, a *cdc28-13* strain that contained cyclin E in place of the *CLN* genes did not grow at 30°C. We speculated that this strain was defective specifically for the START function of the CDC28 protein and not for its G2/M role, presumably because the *cdc28-13* mutation diminished its ability to interact productively with cyclin E. These properties of the *cyclin E-cdc28-13* strain are shown in FIGURE 5. We used this strain as a host to screen for human genes that could interact with cyclin E to perform START. Unlike screens requiring human genes to completely substitute for *CDC28*, this screen may not require that the human genes function at G2/M. We transfected this strain with the human cDNA expression library described above, and 10^5 independent colonies were tested for their ability to grow on glucose. We identified five yeast clones whose growth depended upon expression of both the human cDNAs (*cyclin E* and the new one) (FIGURE 6). The human cDNA within each of these clones was restriction mapped (data not shown). Four of these (S2-6a2, S2-103, S2-112, S2-227) contained the *CDC2-HS* gene (Lee & Nurse, 1987). The *S. pombe cdc*$^+$ gene also performed START together with human *cyclin E* in this strain (F.C. & A. Tinkelenberg, unpublished observations). These results provide further evidence that the cyclin E protein controls START through its interaction with the CDC2 or CDC28 protein. The fact that the *S. cerevisae CDC28* and *CLN* genes can be

replaced simultaneously by human proteins also emphasizes the extent to which the basic cell cycle machinery has been conserved in evolution.

EXAMPLE 6

Transit through START in yeast containing human *cyclin E* and human or *Xenopus CDK2*, a member of the *CDC2* gene family

The restriction map of the fifth clone, S2-124, did not match that of *CD2-HS*. Recently, the human homolog of the *Xenopus CDK2* gene (formerly called *Eg-1*; Paris et al., 1991) was cloned (S. Elledge, personal communication), and the restriction map of S2-124 matched that of *CDK2-HS*. In addition we found that the *Xenopus CDK2* gene also substituted for *CDC28* and performed START in conjunction with cyclin E. Therefore, humans and *Xenopus* contain at least two members of the *CDC2* gene family that can control the G1/S transition in yeast. In order to test whether the human *CDC2* gene, or the *CDC2* homolog *CDK2*, fully complemented *cdc28-13*, we grew these transformants at 38°C (FIGURE 5). At 38°C *cdc28-13* is defective compared to wild-type *CDC28* for both G1/S and G2/M (Reed & Wittenberg, 1990). As expected (Wittenberg & Reed, 1989) cells containing the *CDC2-HS* gene grew equally well at 30° or 38°C, showing that the *CDC2-HS* gene was able to complement both the G1/S and G2/M functions of *cdc28-13*. Curiously, at 38°C the strains containing the human or *Xenopus CDK2* gene and human *cyclin E* failed to grow. Therefore, under these experimental conditions, the human or *Xenopus CDK2* genes only partially substituted for *CDC28*. In addition, initial attempts to complement a *GAL-CDC28* strain with *CDK2-HS* (on glucose) showed that complementation was extremely poor compared with complementation by *CDC2-HS*. The failure of *CDK2-HS* to fully complement either *cdc28-13* or *GAL-CDC28* is consistent with a previous report showing that the *Xenopus CDK2* gene did not complement either *cdc28* or *cdc2* temperature-sensitive alleles (Paris et al., 1991). The explanation for the partial rescue of *cdc28-13* by *CDK2* is unclear, but one possibility is that the CDK2 protein can complement effectively the G1/S but not the G2/M function of *CDC28*. To address this issue definitively, it would be essential to determine the cell cycle arrest points of the strains described above. This has not been possible because the instability of the plasmids resulted in only a minority of the cells containing all three plasmids even on selective medium (data not shown).

EXAMPLE 7

Activation of human p34 CDC2 by cyclin E

Mixing cyclin E protein with G1 cell extracts demonstrated directly that cyclin E could bind the human CDC2 protein *in vitro* and that this association led to activation of the CDC2 kinase. We have shown previously that human G1 cells contain no active p34 CDC2 kinase; all the P34 protein present in the cell is monomeric, unassociated with any cyclin (Draetta & Beach, 1988; D'Urso et al., 1990). G1 extracts were prepared from MANCA cells, a human Burkitt's lymphoma cell line. We confirmed that these G1 extracts contained no detectable CDC2 kinase. The extracts were mixed with a vast excess of p13-Sepharose relative to CDC2 protein, conditions that ensure quantitative binding of CDC2 protein to p13-Sepharose. No histone H1 kinase activity could be detected specifically associated with the p13-Sepharose beads (see FIGURE 7B). Also, these G1 extracts were inactive in a kinase assay using a specific peptide substrate of the CDC2 kinase (Marshak et al., 1991).

To study the interaction between cyclin E and CDC2, cyclin E was expressed in *E. coli* as a glutathione transferase fusion protein (GT-cyclin E) and purified by affinity chromatography on glutathione-Sepharose. We incubated the G1 cell extract with GT-cyclin E-Sepharose, GT-Sepharose, p13-Sepharose, and blank Sepharose beads. GT-cyclin E-Sepharose and p13-Sepharose bound equivalent amounts of p34 CDC2 protein, as detected by immunoblotting using a C-terminus-specific p34 CDC2 antiserum (FIGURE 7A). We detected no binding of p34 CDC2 to GT-Sepharose or blank Sepharose.

After incubation in the G1 extract the Sepharose beads were assayed for histone H1 kinase activity. Only the GT-cyclin E-Sepharose beads contained histone H1 kinase activity, even though the p13-Sepharose and GT-cyclin E-Sepharose beads bound equal amounts of p34 CDC2 protein (FIGURE 7B). We also observed that the cyclin E fusion protein was phosphorylated by the bound kinase. A protein precisely comigrating with the cyclin E fusion protein was phosphorylated during the H1 kinase reaction, and this phosphoprotein was immunoprecipitated by a cyclin E antiserum (FIGURE 7B). Cleavage of the phosphorylated GT-cyclin E fusion protein with thrombin showed that the cyclin E portion of the fusion protein was phosphorylated (data not shown). Phosphorylation of the GT-cyclin E fusion protein was probably due to the bound CDC2 kinase (see below), since autophosphorylation of the cyclin subunit is characteristic of cyclin-CDC2 complexes (Draetta & Beach, 1988; Pines & Hunter, 1989).

The experiments described above did not demonstrate directly that the GT-cyclin E-associated kinase was the p34 CDC2 kinase. To test this, we released the GT-cyclin E-associated proteins from the Sepharose beads by incubation with free glutathione. The released p34 CDC2 protein was immunoprecipitated with a C-terminus-specific p34 CDC2 antiserum and shown to have histone H1 kinase activity (FIGURE 7C). As a control we showed that no kinase was immunoprecipitated from the protein released from the GT-Sepharose beads (FIGURE 7C). We do not know what fraction of the GT-cyclin E-bound kinase could be immunoprecipitated with the p34 CDC2 antiserum and therefore cannot rule out that other kinases (such as CDK2) contributed to the GT-cyclin E-bound kinase activity.

Our results show that cyclin E bound the p34 CDC2 kinase and support the idea that it was activated by cyclin E. The fact that no CDC2 kinase was detected in the initial G1 extract suggests that association of the CDC2 protein with the GT-cyclin E-Sepharose led to activation of previously inactive protein. Since these experiments were done in crude cell extracts, they could not address whether the association of cyclin E with CDC2 protein was sufficient for activation of the CDC2 kinase. Additional modifications of either the CDC2 or cyclin protein may be necessary steps in the activation pathway.

Antibodies were raised in rabbits against the GT-cyclin E fusion protein. These antibodies specifically recognized cyclin E, as they immunoprecipitated *in vitro* translated cyclin E, but not human cyclin A or B (FIGURE 8A). This antiserum immunoprecipitated an H1 kinase activity from HeLa cells (FIGURE 8B). This suggested that cyclin E-associated with a kinase *in vivo*, although we do not know which members of the CDC2 family were present in these complexes.

EXAMPLE 8

Cell cycle dependent activation of cyclin E and cyclin A-associated protein kinases

The previous Examples show that immunoprecipitates of cyclin E from exponentially growing MANCA cells (a human B cell line) contain a cell division kinase. Cyclin E-associated kinase activity during the cell cycle was investigated using centrifugal elutriation to separate exponentially growing MANCA cells into 8 fractions. Centrifugal elutriation physically separates cells into different cell cycle fractions thereby avoiding potential artifacts known to be associated with induced synchronization procedures. We determined the position of an elutriated fraction by measuring the nuclear DNA content by flow cytometric analysis of propidium iodide

stained nuclei (FIGURE 9A). Cellular extracts from each of the eight different cell cycle fractions were immunoprecipitated using an anti-cyclin E polyclonal antiserum. Pre-immune serum (αPI) from the same animal .was used as a negative control. For each fraction, the kinase activity in the control immunoprecipitates was subtracted from the activity observed in the specific anti-cyclin E immunoprecipitates. The data, presented in FIGURE 9B, show the level of expression of cyclin E-kinase complexes in the elutriated fractions of cells (Figure 9A) as determined by measuring the level of histone H1 phosphorylation catalyzed by cyclin E-associated H1 kinase activity. Equal numbers of cells from each fraction were lysed, and cyclin E in the lysates was immunoprecipitated with affinity purified anti-cyclin E antibodies (αcycE). The results, quantitated by phosphor-imaging, indicate that cyclin E-associated kinase activity was cell-cycle-dependent and, in three experiments, fluctuated during the cell cycle by between 4- and 8-fold. The peak in cyclin E-associated kinase activity corresponded to elutriated fractions of cells having the greatest number of cells in late G1 and early S phase. In some experiments, we also observed a smaller second peak of cyclin E-associated kinase activity in the G2/M fraction of elutriated cells (data not shown).

The activity of the cyclin E-associated kinase during the cell cycle is markedly different from the kinases associated with cyclin A. C160 anti-cyclin A monclonal antibodies were used to immunoprecipitate cyclin A and its associated proteins from the same cell extracts that had been used to measure cyclin E-associated kinase activity (FIGURE 9C). As previously described, cyclin A-associated kinase activity is first detected at the start of S phase (Pines and Hunter, 1990; Marraccino et al., 1992). In contrast to cyclin E-associated kinase activity, the cyclin A-associated kinase activity continues to rise throughout S phase and peaks in G2. These results also indicate that peak levels of the cyclin A-associated kinase are approximately 5 to 10-fold greater than the peak activity levels of the cyclin E-associated kinase (data not shown). However, the absolute levels may vary since the levels presented here depend upon two antibodies that may have slightly different association constants (K_a). These results suggest that a succession of distinct cyclin dependent kinase activities are activated during the cell cycle; kinase activity of cyclin E increases, followed by an increase in cyclin A- then cyclin B-associated kinase activity.

The kinetics with which the cyclin E-associated kinase activity accumulate during the G1 phase of the cell cycle was investigated as a relative measure of the abundance of the enzymatically active cyclin E:kinase complex. MANCA cells were arrested for 3 hours in the metaphase stage of the cell cycle with nocodazole at which

time 75% of the cells had completed cytokinesis. Cells seperated from residual mitotic cells by elutriation were then released into the G1 phase of the cell cycle for 3,4,5,6 or 7 hours. They progressed synchronously into S phase after about 6 or 7 hours as determined by both flow cytometric measurement of nuclear DNA content (9D) and tritiated thymidine incorporation into chromosomal DNA (data not shown). Cells were then fractionated into sub-populations in different phases of the cell cycle by centrifugal elutriation. Cyclin E-associated kinase activity was found to be elevated during the G1 period reaching peak activity just as the cells entered S phase (FIGURE 9D). In contrast, we found that cyclin A-associated kinases were not present in G1 and were first detected as cells entered S phase (FIGURE 9E). In this experiment, cyclin A-associated H1 kinase activity was determined using C160 anti-cyclin A monoclonal antibodies to imminoprecipitate cyclin A-associated kinase activity. The elutriated G1 fraction of cells (fraction 2) was cultured at 32.5°C to expand the G1 phase of the cell cycle. Aliquots of cells were harvested hourly for measurement of nuclear DNA content, and cyclin A- and cyclin E-associated kinase activities, up to the point where the cells approached and entered S phase.

Cyclin E-associated kinase is readily detectable in proliferating rat 208F cells but disappears when they enter quiescence after serum withdrawal (FIGURE 10A). Similarly, when rat PC12 cells were induced to differentiate into neurons by exposure to NGF, the cyclin E-associated kinase fell to low levels (FIGURE 10B). In these experiments, we assayed H1 kinase activity in lysates from growing and quiescent rat 208F cells, and growing rat PC-12 cells. Immunoprecipitates were prepared using affinity-purified antibodies against cyclin E (αE), the C-terminus of human p34 CDC2 (αp34), or as a control pre-immune anti-cyclin E antiserum (αPI). Cells were induced to grow using 10% calf serum (10%CS, FIGURE 10A) and to differentiate using NGF (+NGF, FIGURE 10B). Quiescent controls were grown in 0.1% calf serum (0.1%CS, FIGURE 10A). Nondifferentiating controls were grown in the absence of NGF (-NGF, FIGURE 10B). These results demonstrate that cyclin E-associated kinase activity is growth regulated as are the levels of *cyclin E* expression.

EXAMPLE 9

Constitutive expression of cyclin E shortens G1

The pattern of cyclin E-associated kinase activity during the cell cycle suggested that the physiological function mediated by cyclin E takes place during the G1 phase of the cell cycle. To test this possibility, stable cell lines were constructed that constitutively expressed human *cyclin E* from a retroviral LTR promoter. The

effects of constitutive *cyclin E* expression on cells, and on cell cycle kinetics was tested.

A human *cyclin E* cDNA was cloned into the retroviral expression vector, LXSN (Miller & Rosman, 1989), which expresses the inserted cDNA from the 5' LTR and contains the neomycin phosphotransferase gene as a selectable marker. The construct permits production of retroviral vector particles having either amphotropic or ecotropic host range specificity. We used an ecotropic stock of vector particles to infect the Rat-1 fibroblast cell line and selected a pool of over ten thousand independently infected cells by growth for 2 weeks in G-418 containing selective media. At the same time, a pool of LXSN control vector-infected cells was generated. Pools of infected cells were studied rather than individual selected cell clones to minimize any possible clonal variation that might be present within the Rat-1 cell line. The results, presented in FIGURE 11A(a), show that the LXSN-*cyclin E* infected cells contained approximately 5 to 10-fold more cyclin E protein than could be detected in the cells infected with the control LXSN viral vectors. Two cyclin E bands, at 45 kDa (the size of full length cyclin E) and 40 kDa, were specifically expressed in *cyclin E* transduced cells. Increased cyclin E:kinase activity was also observed in the LXSN-*cyclin E* transduced cells. The results in FIGURE 11A(b) show a 3 to 5-fold increase in the level of cyclin E-associated histone H1 kinase activity in exponentially growing cultures of Rat-1 cells. The lower levels of cyclin E and cyclin E-associated kinase detected in the control cells was presumably due to endogenous rat cyclin E. To evaluate the effects of *cyclin E* on the cell cycle, exponentially growing LXSN-*cyclin E* transduced cells were collected by centrifugal elutriation. The distribution of transduced cells within the cell cycle was determined by flow cytometry after DNA staining with propidium iodide (FIGURE 11B). Cells transduced with LXSN-*cyclin E* showed a decrease in the fraction of cells in the G1 phase of the cell cycle in comparison to control cells transduced with the control vector, LXSN. The *cyclin E* transduced cells also showed an increase in the fraction of cells in the S phase of the cell cycle. These observed changes in the fractions of *cyclin E* transduced cells in the different phases of the cell cycle are consistent with accelerated transit of the cells through the G1 phase of the cell cycle. This was confirmed by directly measuring the length of the G1 phase in cyclin E infected cells. *Cyclin E* infected cells were synchronized in pseudometaphase by exposure to nocodazole, and mitotic cells collected. The mitotic cells were returned to culture and entry into S phase was monitored by pulse labeling with BrdU (5-bromodeoxyuridine). The BrdU was detected using immunochemical methods. The

results, presented in FIGURE 11C, show that the length of the G1 phase in the LXSN-*cyclin E* infected cells (i.e., from conclusion of mitosis until resumption of DNA synthesis in S-phase) was substantially shorter than in cells transduced with LXSN.

In 5 separate experiments, using two independent pairs of transfected cell populations, the duration of G1 was, on average, 33% shorter in cells infected with the LXSN-*cyclin E* retroviral vector than in cells infected with the LXSN control vector. Similarly, studies conducted to measure the rate of entry of *cyclin E*-transduced cells into S phase using immunochemical detection of BUDR incorporated into nuclear DNA confirmed shortening of the S phase in LXSN-*cyclin E* transduced cells (data not shown). Due to the limited number of cells that can be obtained by mitotic shake-off methods, it was not possible to measure the level of cyclin E-associated kinase activity at each time point during progression from mitosis to S phase in the infected Rat-1 cell populations.

EXAMPLE 10

Cyclin E-associated proteins

Previous Examples have shown that cyclin E can activate human p34 CDC2, and human or *Xenopus* p33 CDK2, when the proteins are expressed together in budding yeast. Furthermore, the results have shown that cyclin E can bind and activate both human CDC2 and human CDK2 *in vitro* (i.e., in cell-free systems). The association of kinases with cyclin E was examined in *in vivo* studies, (i.e., in cells), where cell extracts were prepared from elutriated cell cycle fractions of exponentially growing MANCA cells that were biosynthetically radiolabeled with [^{35}S]-methionine for 3 hours. Extracts were prepared in SDS-RIPA buffer and the specific proteins were immunoprecipitated using affinity purified anti-cyclin E antibodies (prepared against GST-cyclin E fusion protein), and an antiserum against the C-terminus of human CDC2 (p34). Immunoprecipitates were collected, washed, and boiled in the SDS buffer prior to separation on 12% SDS-polyacrylamide gels. Detection of radiolabeled polypeptides in the gels was facilitated using sodium salicylate; and the gels were then dried for autoradiography. FIGURE 12 shows the p34-associated proteins (Lane 1) and the proteins associated with cyclin E (Lanes 2-11) in exponentially growing cells (Lane 2), during G1-phase (Lanes 3), G1/S (Lane 4), S (Lanes 5-8), S/G2 (Lanes 9-10) and M-phase (Lane 11). The molecular weights of the proteins associating with the cyclin E:CDC kinase complexes in the assay of FIGURE 12 are summarized in Table 1, below. The 13Kd polypeptide was associated with the complex during the middle of S-phase; the 17Kd, throughout the

cell cycle; the 32Kd-doublet, mostly late in G1 and S; the 36Kd, only in G1 and G2/M; the 70Kd, predominantly in S; the 85Kd, (i.e., the lowest band in the triplet) throughout the cell cycle; and, the 107Kd, in S-phase and just before and after S-phasse. The difference in the expression pattern of the 32Kd-double suggested that it was not CDK2 or CDC2, and this was confirmed by mapping the tryptic fragments of CDK2, CDC2, and the 32kD band, designated band "x", associated with the immunoprecipitated complexes.

Table 1

Molecular Sizesof Cyclin E:CDC kinase Accessory Proteins

		Presence of Band with Apparent Molecular Size (Kd)[c]						
Immunoppt.[a]	Cell Cycle Phase[b]	13	17	32	36	70	85	107
anti-p34	Exponential	+/-	+	-	+/-	-	-	-
anti-cyclin E	Exponential	+/-	+	2+	+/-	2+	+	+/-
	G1	+	+	+	+	+	+	-
	G1/S	+	+	2+	+/-	2+	+	+
	S	2+	+	3+	+/-	3+	3+	+
	S/G2	2+	2+	3+	+/-	2+	3+	+
	G2/M	+	2+	3+	2+	+	3+	+/-

a.) Immunoppt.= immunoprecipitate prepared with anti-p34 CDC2 or anti-cyclin E;
b.) Cell cycle phase, centrifugal elutriation fractions of cells; and,
c.) Molecular size in kilodaltons of polypeptides associated with cyclin E:CDC kinase complexes. 32kd= middle of doublet, 85kd= lowest band of triplet; amount indicated on a scale from - to 3+.

A series of control immunoprecipitaton reactions were conducted to characterize the specificity of anti-CDC2 and anti-CDK2 antibodies. Lysates from exponentially growing MANCA cells were immunoblotted using affinity purified antibodies directed toward the 7 C-terminal amino acids of human p34 CDC2 (α CDC2) or antiserum directed toward the 15 C-terminal amino acids of human p33 CDK2 (αCDK2). In FIGURE 13, lanes 1 and 2 are immunoblots of whole cell extracts; in lanes 3 and 4, whole cell extracts were first immunoprecipitated with affinity purified anti-p34 CDC2 antibodies and then blotted with the indicated antibodies; in lanes 5 and 6, extracts were first immunoprecipitated with an antiserum against the C-terminus of p33 CDK2 and then blotted with the indicated antibodies. Note the presence of a non-specific signal derived from the immunoprecipitating antibody between 50 and 80 kDa. An extract from MANCA cells arrested at the G1/S boundary with aphidicolin was immunoprecipitated using affinity purified antibodies against human cyclin E and then blotted using the same antibodies. A single protein band at 45 kDa was detected. Therefore, the associated proteins in

cyclin E immunoprecipitates were most likely bound to cyclin E and were not detected due to nonspecific cross-reactivity with this antibody.

To confirm these results, immunoblotting was used to examine the association between cyclin E and both p33 CDK2 and p34 CDC2 in extracts prepared from MANCA cells growing exponentially or arrested at the G1/S boundary with aphidicolin. The aphidicolin blocked cells were chosen because the activity of the cyclin E-associated kinase is maximal at the G1 to S phase transition. Cell extracts were immunoprecipitated using affinity-purified anti-cyclin E antibodies and the immunoprecipitates western blotted using both CDC2 and CDK2 specific antisera. For all immunoprecipitations the antibodies had been cross-linked to sepharose. Immunoprecipitations were carried out with pre-immune serum ("αPI"), blank sepharose beads ("SEPH"), affinity purified anti-p34 CDC2 C-terminus ("αp34"), and affinity purified anti-cyclin E ("αE") (FIGURE 14A). The set of lanes labeled "--" contained no cell extract. Both antisera were raised against peptides corresponding to the C-termini of the respective proteins. The C terminus of the CDC2 related proteins is not highly conserved. The results show that the anti C-terminus CDC2 antiserum recognized CDC2 and not CDK2, and conversely that the anti-C-terminus CDK2 antiserum recognized CDK2 and not CDC2 (FIGURE 13).

Immunoblots of whole cell extracts show two forms of CDK2 (Rosenblatt et al., 1992; see also FIGURE 14A). In both aphidicolin arrested cells (FIGURE 14A) and in exponentially growing cells (not shown; see FIGURE 15) cyclin E preferentially associated with a more rapidly migrating form of CDK2. The identification of CDK2 in cyclin E immunoprecipitates has been confirmed using 3 different antisera independently raised against the C-terminus of human CDK2. All three antisera recognize CDK2 and not CDC2 (Rosenblatt et al., 1992; Elledge et al., 1992; FIGURE 13). The more rapidly migrating forms of CDK2 are currently believed to be more highly phosphorylated (Rosenblatt et al., 1992). This is consistent with our observation that all the cyclin E-associated isoforms of CDK2 detected in [^{35}S]-methionine-labeled cell extracts were also detected in anti-cyclin E immunoprecipitates from [^{32}P]-orthophosphate-labeled cell extracts.

The results show that p34 CDC2 was also detected in the cyclin E immunoprecipitates although its abundance was substantially less than that of CDK2. In exponentially growing cells, a predominantly hypophosphorylated form of p34 CDC2 was detected, while in aphidicolin-arrested cells there were also more highly phosphorylated forms of p34 CDC2 associated with cyclin E (FIGURE 14B). In both

cases, it was possible to detect only very small amounts of p34 CDC2 associated with cyclin E.

EXAMPLE 11

Cell cycle dependent formation of a cyclin E:CDK2 complex

The phase in the cell cycle at which cyclin E and CDK2 form an enzymatically active complex was investigated. Exponentially growing MANCA cells were separated into 8 cell cycle fractions by centrifugal elutriation and cellular extracts prepared. The cell cycle position of the cells in each fraction was determined by flow cytometric measurement of nuclear DNA content (FIGURE 15A). Cyclin E and its associated proteins were immunoprecipitated using affinity-purified anti-cyclin E antibodies. We visualized the presence of CDK2 by Western blotting using an antiserum specific for the C-terminus of CDK2 (FIGURE 15B1-B4). The results show that the level of enzymatically active cyclin E:CDK2 complex peaked during late G1 and early S phase and declined in abundance as cells progressed through the remainder of the cell cycle. The abundance of the cyclin E:CDK2 complex closely corresponded to the cell cycle periodicity of the cyclin E-associated kinase (as described in prior Examples). Furthermore, the present results suggest that in exponentially growing cells, cyclin E:CDK2 complexes did not accumulate in an inactive form prior to their activation in late G1. This pattern of appearance and activation was observed to be similar to the pattern reported for cyclin A-associated kinase activity, i.e., the activity of which reportedly increased in direct proportion to the abundance of cyclin A (Pines and Hunter, 1990; Marracino et al., 1992). However, the present results were different from those obtained with the cyclin B:p34 CDC2 complex in that the cyclin B:CDC2 complex reportedly accumulates during S and G2, inactive and highly phosphorylated, prior to their activation at the onset of mitosis (Gould and Nurse, 1989; Pondaven et al., 1990; Solomon et al., 1990).

EXAMPLE 12

Abundance of cyclin E is cell cycle regulated

The abundance of the cyclin E protein was determined at different phases of the cell cycle. MANCA cells were separated into fractions representing each stage of the cell cycle by centrifugal elutriation. We analyzed cell lysates from each fraction by immunoprecipitation using affinity-purified anti-cyclin E antibodies which we also used to measure the abundance of cyclin E in the immunoprecipitates. The results showed that cyclin E levels were maximal in late G1 and declined in S, G2 and M (FIGURE 15B1-B4). The immunoassay procedure was found to accurately reflect

the relative levels of cyclin E in each cell cycle fraction since the amount of cyclin E protein detected was linearly dependent on the amount of cell extract used in the immunoprecipitation (FIGURE 15C). In sum, these results suggest that the abundance of the cyclin E:CDK2 complex, and hence the periodicity of the cyclin E-associated kinase activity, may be directly regulated by the level of cyclin E.

EXAMPLE 13

Assembly of cyclin E:CDC2 and cyclin E:CDK2 complexes *in vitro*

As shown, cyclin E preferentially associates with p33 CDK2 rather than p34 CDC2 in human cells. One possible explanation for this is that the affinity of cyclin E is different for CDK2 than for CDC2. This possibility was evaluated in a cell-free system of recombinant cyclin E and cell extracts containing CDC2 and CDK2 kinases. *Cyclin E* was expressed in Sf9 insect cells using baculovirus vectors. Cyclin E protein were over-expressed in the transduced insect cells, the intracellular concentrations was approximately 5-10 μM after 48 hours (Desai et al., 1992), and these cells were harvested and proteins extracted for analysis. The binding between cyclin E, CDC2, and CDK2 was evaluated using diluted insect cell extracts as a source for cyclin E, and extracts from G1 cells as a source of CDC2 and CDK2. To determine cell-cycle-dependent differences in the effects of cyclin E on the CDC2 and CDK2 kinases, cell extracts were prepared from cells whose growth was arrested for 12 hours (i.e., prior to S-phase) in media containing 2mM hydroxyurea (causing cells in S-phase to stop and all other cells to pile up next to S-phase) followed by release of growth for 3.5 hours to allow all cells to enter S-phase ("HU" FIGURES 16A-16B); as well as, from cells blocked with nocodazole, released for three hours into G1, and then further selected by centrifiugal elutriation ("G1", FIGURES 16A and 16B). All cell extracts were prepared in hypotonic buffer. The incubation mixtures were designed to bring the concentration of the three proteins close to the normal physiologic levels at approximately 0.2 μM. Diluted lysates containing the indicated cyclin E, CDC2, and CDK2 proteins were incubated alone or in combination for 30 minutes at 37°C under conditions suitable for *in vitro* replication of SV40 origin containing plasmids (D'Urso et al. 1990). The formation of cyclin E:CDC2 and cyclin E:CDK2 complexes in the incubation mixtures was determined using immunoprecipitation either with antisera to CDC2 (anti-CDC2), the C-terminus of CDK2 (anti-CDK2), or cyclin E (anti-cyclin E) followed by SDS-PAGE, and autoradiography (FIGURE 16A, 16B, 16C). The kinase activity associated with the different respective immunoprecipitates was determined in the H1 kinase assay (as described in the Examples, above).

The immunoprecipitates were tested for their ability to mediate phosphorylation of histone H1 (i.e., H1 kinase activity) by mixing the immunoprecipitates with histone H1 and γ-^{32}P orthophosphate. ^{32}P-radiolabeled histone H1 was detected by SDS-PAGE and phosphor imaging (FIGURE 16A, 16B, 16C). (The phosphor imaging in FIGURES 16A-16C was quantified and the results are graphically presented in FIGURE 17.) CDC2 kinase activity, while evident at low levels in immunoprecipitates prepared from HU-arrested cells (FIGURE 16C, "HU"), was decreased to nearly undetectable levels during G1-phase (FIGURE 16C, G1 extract, "0") and the level of kinase activity was not altered by addition of different amounts of cyclin E to the cell extract (i.e., FIGURE 16A, 16B, 16C; "5, 1, 0.2"). In contrast, CDK2 kinase activity present at low levels in HU-arrested cell extracts (FIGURE 16A, "HU"), decreased to undetectable levels in G1 (FIGURE 16A, G1 extract, "0"), but when cyclin E was added to the G1 cell extract (FIGURE 16A, "5,1,0.2") the CDK2 kinase activity was restored. The results show activation of a latent CDK2 kinase activity in the G1-phase cell extracts following addition of cyclin E, and suggest that kinase activity is regulated by the abundance of cyclin E. Quantitive aspects of these studies are presented in FIGURE 17, where the level of cyclin E-mediated activation of CDK2 kinase activity was measured (i.e., using phosphor imaging of the SDS-PAGE gels presented in FIGURES 16A, 16B, and 16C, above) as a function of the amount of cyclin E added to the G1 phase cell extract ("fold cyclin E in HU extract"; FIGURE 17). (The differing amounts of Sf9 lysate containing cyclin E in FIGURE 17 correspond to the "5, 1, and 0.2" amounts in FIGURE 16A, 16B, and 16C.) The phosphor imaging data for the kinase activity of each of the CDC2, CDK2, and cyclin E immunoprecipitates was normalized by calculating the activity as a percentage of the activity seen in cell lysates of HU-arrested control cells (i.e., 100%; "%hydroxyurea H1 kinase"; FIGURE 17). The results presented in FIGURE 17 show that the level of CDK2 kinase activity was dependent upon the amount of cyclin E added to the G1 extract, and that levels of CDK2 kinase activity were achieved which were more than 22-fold greater than those observed in the HU-arrested cell extracts (i.e., cyclin E immunoprecipitate at 5-fold cyclin E; FIGURE 17). In addition, the results show that the kinase activity associated with the cyclin E-immunoprecipitates was consistently greater than that associated with CDC2 immunoprecipitates. The results also confirm the previous findings (above) that only low levels of CDC2 activity are present in the G1-phase cell extracts, and that any latent CDC2 that might be present in these extracts is not appreciably activated by the addition of cyclin E.

These combined results suggest activation of kinase activity by cyclin E resulting from formation of a cyclin E:CDK2 complex. In other studies (not shown) the association of cyclin E with CDC2 or CDK2 was verified using molecular-sieve gel chromatography on Superose 12. p34 CDC2 and p33 CDK2 monomers eluted at 30-40 kDa and had negligible histone H1 kinase activity. When insect cell extracts containing recombinant cyclin E were mixed with the CDK2-containing lysate, the majority of the CDK2 protein eluted at an approximate molecular size of 160 kDa, suggesting formation of a cyclin E:CDK2 complex. In contrast, when extracts containing a similar amount of CDC2 were mixed with the cyclin E lysate only a small fraction of CDC2 protein associated in a stable manner with cyclin E. The cyclin E:CDC2 and cyclin E:CDK2 complexes eluted from the molecular sieve column exhibited kinase activity.

<u>Discussion of Examples 8-13</u>

<u>Cyclin E is a G1 Cyclin</u>.

The proliferation of eukaryotic cells is primarily regulated by a single decision which occurs during the G1 phase of the cell cycle--either to enter the cell cycle and divide or to withdraw from the cell cycle and enter a quiescent state (reviewed in Baserga, 1985; Pardee, 1989). In yeast, the biochemical process that underlies this cellular decision is the assembly and activation of a complex between the CDC8 protein kinase and the CLN type cyclins (reviewed in Nurse, 1990; Hartwell, 1992). Recent experiments in a variety of model systems support the idea that the role of the CDC2-related kinases have been evolutionarily conserved (D'Urso et al., 1990; Blow-& Nurse, 1990; Furakawa et al., 1990; Fang-& Newport, 1991). The observations presented here demonstrate that human cyclin E specifically activates a CDC2 related kinase during the late G1 phase of the cell cycle and that cyclin E accumulation is rate-limiting for G1 transit. Therefore, we suggest that in all eukaryotes a critical step in the biochemical pathway that controls cell proliferation is the assembly of a cyclin/CDK complex (the term CDK is used to designate a cyclin dependent kinase in the CDC2 protein family).

The evidence that cyclin E functions during the G1 phase of the human cell cycle can be summarized as follows: Cyclin E can perform the G1 START functions of the yeast CLN proteins since it can complement mutations in the yeast *CLN* genes (Koff et al., 1991; Lew et al., 1991). Furthermore, we have shown that cyclin E in combination with either human CDC2 or human CDK2 could rescue yeast strains that were doubly mutated for both CLN and CDC28 function (Koff et al., 1991). However, the specificity of the assay was suspect since human cyclin B, which clearly

functions during mitosis and not during G1, could also rescue *CLN* mutations (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991). As reported here, cyclin E associates with a protein kinase in human cells and this kinase is cell cycle regulated. The activity of the cyclin E-associated protein kinase, as well as the abundance of the cyclin E protein, peaks during late G1 and early S phase, and then declines as cells progress through S, G2 and mitosis. This kinase is also growth regulated, since it is absent from cells that have exited the cell cycle and differentiated or become quiescent. The relative timing of cyclin E and cyclin A activity is significant.. Cyclin A protein and cyclin A-associated kinase activity are detectable as soon as S phase starts (Marraccino et al., 1992), and cyclin A function is necessary for S phase to being (Girard et al. 1991). We have also shown that cyclin E accumulates before cyclin A and that the cyclin E-associated kinase appears earlier in the cell cycle than the kinase associated with cyclin A. This biochemical function of cyclin E during G1 suggested that its physiological function would precede the S phase role of cyclin A. This was directly shown by constitutively expressing human *cyclin E* in the rat fibroblast cell line, Rat-1. We found that 5 to 10-fold overexpression of *cyclin E* caused a 3 to 5-fold increase in the level of cyclin E-associated kinase activity. This level of *cyclin E* overexpression caused a 30-35% decrease in the length of the G1 phase of the cell cycle.

The abundance of the cyclin E protein is normally cell cycle regulated--it shows a sharp peak in late G1. This is probably due to regulation of the cyclin E mRNA level (Lew et-al., 1991) since it fluctuates during the cell cycle in parallel with the level of the cyclin E protein. The mRNA's encoding cyclin E, A and B are cell cycle regulated and predict the pattern of accumulation of the respective cyclin proteins (Pines and Hunter, 1989, 1990). In budding yeast, accumulation of the CLN mRNA's is under positive feedback control and result in a rapid rise in CLN mRNA and protein levels at START (Cross and Tinkelenberg, 1991; Dirick & Nasmyth, 1991). The association of cyclin proteins with transcription factors in mammalian cells may be part of an analogous mechanism that controls the timing of cyclin gene expression during the cell cycle (Bandara et al., 1991; Mudryj et al., 1991; DeVoto et al., 1992; Shirodkar et al., 1992). While cyclin accumulation is in part determined by the levels of the respective mRNA's, cyclin abundance can also be controlled by protein turnover (Murray & Kirschner, 1989; Glotzer et al., 1991). It is not known whether the stability of the cyclin E protein is regulated during the cell cycle, but the protein lacks the consensus sequence recognized by the ubiquitinating enzyme that mediates the mitotic turnover of cyclins A and B (Glotzer et al., 1991).

The Cyclin E:CDK2 complex

The data suggest that the major cyclin E-associated protein kinase is CDK2. Two dimensional gel analyses of ^{32}P or ^{35}S-met labelled proteins show that the major CDC2-related protein associated with cyclin E in human cells is CDK2. While there is not direct evidence that the cyclin E:CDK2 complex is an active kinase *in vivo*, this is the most likely conclusion. The abundance of the cyclin E:CDK2 complex is cell cycle regulated and closely parallels the levels of the cyclin E-associated kinase. Furthermore, the CDK2 protein bound to cyclin E is primarily in the more rapidly migrating of the two forms detectable by one dimensional PAGE. This downward mobility shift is known to correlate with both binding of CDK2 to cyclin and activation of the CDK2 kinase (Rosenblatt et al., 1992). It is thought to be indicative of phosphorylation of threonine 160, which is a prerequisite for activation of the CDK2 kinase (Y. Gu and D.M., unpublished observations). It has also been shown that the cyclin E:CDK2 complex can substitute for the CLN/CDC28 complex in *S. cerevisae* (Koff et al., 1991), and that the cyclin E:CDK2 complex is an active kinase *in vitro*.

The periodic accumulation of the cyclin E protein in cells appears to match that of the cyclin E:CDK2 complex, whereas the CDK2 protein is present at invariant levels during the cell cycle (Rosenblatt et al., 1992). Therefore, it would seem that the abundance of the cyclin E:CDK2 complex is primarily regulated by the level of the cyclin E protein. However, the phosphorylation state of cyclin E could also control the assembly of the complex.

At least six phosphorylated isoforms of CDK2 are associated with cyclin E. This complexity was surprising since only two of these isoforms had been detected bound to cyclin A. Preliminary evidence indicates that CDK2 is phosphorylated on 3 residues homologous to those phosphorylated in CDC2 (Y. Gu and D.M., unpublished observations)--T14, Y15 and T160. Combinatorial phosphorylation of these sites might account for the six CDK2 isoforms. It seems more likely, however, that other phosphorylation sites are also present since immunoprecipitates with anti-CDK2 antibodies contained two additional phosphorylated isoforms of CDK2, bringing the total number detected to eight (FIGURE 11D). One interpretation is that the cyclin E:CDK2 complex integrates the information provided by the many signals that control cell proliferation, e.g., by binding second messengers involved signal transduction. The multiply phosphorylated forms of CDK2 may reflect the influence of diverse mitogenic signals on the activation of the cyclin E:CDK2 complex. The multiple CDK2 phosphates could have both positive and negative effects on CDK2

activity and a particular phosphorylated state may be required for specific functions. The downstream activation of the cyclin A:CDK2 complex, which occurs after commitment to the cell cycle has been made, may be responsive to much fewer factors and therefore biochemically less elaborate.

Other evidence has indicated that CDK2 might play a role during the G1 of S phases of the cell cycle. In cycling cells, CDK2 kinase activity precedes CDC2 kinase activity (Rosenblatt et al., 1992). Our experiments in *S. cerevisae* showed that in certain genetic backgrounds *CDK2* can complement the G1/S function of CDC28 and not its G2/M functions (Koff et al., 1991). Also, depletion of CDK2 from extracts of activated *Xenopus* eggs prevents the start of DNA replication (Fang and Newport, 1991). All these results are consistent with a role for CDK2 in committing the cell to the cell cycle.

<u>The Cyclin E:CDC2 Complex.</u>

Cyclin E can interact with both human CDK2 and human CDC2 when the proteins are expressed together in yeast and cyclin E can activate both the CDK2 and CDC2 kinases *in vitro* (Examples, above). We have shown that although the cyclin E:CDK2 complex is more abundant in human cells, the cyclin E:CDC2 complex is also present. In addition, complexes between cyclin E and other proteins were observed (FIGURE 12; Table 1) that may potentially modulate or change cyclin E activity. The pattern of cyclin E-associated kinase activity during the cell cycle showed some differences from the abundance of the cyclin E:CDK2 complex. These differences may be attributable to the cyclin E:CDC2 or the other cyclin E complexes.

The low level of the cyclin E:CDC2 complex *in vivo* appears to be a consequence of the relatively low affinity of cyclin E for CDC2. The reconstitution experiments presented here show that the cyclin E:CDK2 complex readily formed under conditions where very little cyclin E bound to CDC2. We have found, however, that cyclin E is present in multiple phosphorylated states *in vivo*. Therefore, another possibility is that only certain relatively rare isoforms of cyclin E can bind to CDC2.

We previously observed that a mutation in the yeast *CDC28* gene greatly curtailed the ability of cyclin E, but not cyclin B, to rescue CLN function and, consequently, we suggested that cyclin E might interact with CDC28 differently than cyclin B (Koff et al., 1991). *In vitro* reconstitution experiments support this idea by showing that cyclin B bound to CDC2 effectively (Desai et al., 1992) while only small amounts of cyclin E:CDC2 complex could be detected.

Other Cyclin E:CDC Complexes:

The results presented in FIGURE 12 and Table 1, above, may also be interpreted to indicate the possible existence of other cell division kinases, previously unrecognized, that associate with cyclin E. The 32Kd band "x" protein (Table 1, FIGURE 12) is certainly a candidate for such a novel kinase protein, both based on the similarity in size with the known CDC2 and CDK2 kinases, and its apparent association with cyclin E.

G1 Regulation in Mammalian Cells

In 1974 Pardee proposed that the proliferation of mammalian cells is regulated by extracellular mitogenic signals at a point during the G1 phase of the cell cycle called the restriction point (Pardee, 1974). If these signals were not present, or if the cell was incapable of appropriately responding to them (e.g. if protein synthesis is inhibited) then the cell would not traverse the restriction point and entered a quiescent state, called G_0 (reviewed in Zetterberg, 1990). While cells can respond to a wide array of extracellular mitogenic signals, one gets the impression that there is much less diversity in the intracellular events triggered by these signals (see Cantley, 1991; Chao, 1992). Indeed, it is not unreasonable to expect that there might be a final common point through which the diverse mitogenic pathways must pass, and that this is the restriction point (Pardee, 1974).

There are few molecular details about restriction point regulation. In normal cells, progression through the restriction point is very sensitive to the rate of protein synthesis (Rossow et al., 1979, Schneiderman et al., 1971; Brooks, 1977). Prior to the restriction point (but not after) small and transient decreases in protein synthesis cause substantially longer increases in the length of G1 (Zetterberg & Larson, 1985). Removal of extracellular mitogenic stimuli and inhibition of cellular protein synthesis, in fact, are thought to deter the same cell cycle event (Pardee et al., 1981). To account for the disproportionately large effect on G1 length by relatively small changes in the rate of protein synthesis, it was proposed that a labile protein must accumulate during G1 in order for the cell to traverse the restriction point (reviewed in Pardee, 1989).

It is appealing to speculate that a cyclin is this labile regulator of the restriction point and that formation and/or activation of a cyclin/CDK complex is a rate-limiting even in G1 progression. The periodic accumulation of cyclin E during the cell cycle indicates that it is a relatively short lived protein, and its G1 peak in abundance may be consistent with a role at the restriction point. Also, the decrease in G1 length by constitutive cyclin E expression suggests that entry into S phase may be

limited by the abundance of cyclin E. It is important to remember, however, that not all of G1 is eliminated by constitutive *cyclin E* expression. Mostly likely, there are some essential G1 events whose duration is not effected by abundance of cyclin E. Examples of this might include chromosome decondensation and nuclear membrane assembly. Furthermore, it has been reported that in some circumstances the G1 restriction point occurs less than one hour before S phase starts (Wynford-Thomas et al., 1985) while in other cases the restriction point can occur much earlier in G1 (Pardee, 1974). Our measurements indicate that the maximal cyclin E-associated kinase levels are reached relatively late in G1. This is particularly apparent in serum stimulated cells where much of G1 is completed before the cyclin E-associated kinase is detected (A.K. and J.R., unpublished observations). Other cyclins, such as *cyclin D* and *cyclin C*, may also be expressed during G1 (Matsushime et al., 1991; Motokura et al., 1991; Lew et al., 1991) and it is possible that the sequential formation of multiple cyclin:CDK complexes is required for the cell to traverse G1. In that case, constitutive *cyclin E* expression might shorten only the latter stages of G1.

In *S. cerevisae* factors that control passage through START can effect CLN function, apparently at multiple levels (Change & Herskowitz, 1990; Cross and Tinkelenberg, 1991). By analogy, we might expect G1 cyclin function in mammalian cells to be controlled by proteins that modulate cell proliferation. For example, it would not be surprising to observe direct interactions between cyclin E and members of the Rb protein family (Bandara et al., 1991; Mudryj et al., 1991; Shirodkar et al., 1992; DeVoto et al., 1992). Also, expression of the cyclin E gene might be regulated by one or more of the oncogenic transcription factors.

EXAMPLE 14

Growth factor-dependence of cells constitutively expressing cyclin E

The cell division cycle of all normal higher eukaryotic cells is controlled by specific extracellular growth factors that are required for cell division. The families of known growth factors is diverse and includes such proteins as insulin, PDGF, IGF, EGF, GM-CSF, G-CSF, TGF, erythropoeitin, and other stem cell factors. Different cell types display particular growth factor requirements, determined in part by the growth factor receptors expressed on their cell surface and by their state of differentiation. Typically, cells in tissue culture require exogenous growth factors in an animal serum (i.e., fetal bovine serum) to grow; or in chemically defined serum-free medium specific growth factors must be added. In the absence of the requisite growth factor(s), cells stop dividing and arrest in G1. The results presented in the Examples above indicated that the level of cyclin E, and/or the activity of the cyclin

E:cell division kinase complex, may be rate-limiting for transit of cells through G1. Therefore, it was reasoned that cell proliferation might be regulated through steps requiring cyclin activation (e.g., increased cyclin gene transcription or translation; or increased cyclin:kinase complex activity) and that growth factors might act upon cells by activating cyclins. Assuming that cyclin activation is required for proliferation two hypotheses were considered: a unitary hypothesis in which a cell at a particular stage of differentiation has a single cyclin that can be triggered by a single growth factor; and a multiform hypothesis, wherein a single growth factor activates multiple cyclins and the combined action of all the cyclins in the cell is required to trigger cell proliferation. It was reasoned that if the simple cause-and-effect logic of the unitary hypothesis held true, then modifying cyclin E levels in a cell might alter the growth factor requirements of the cell for proliferation *in vitro*; while if the multiform hypothesis held true, then any alteration in a single cyclin might be masked by the action of all the other cyclins in the cell. To test these two hypotheses, cells were transduced with the LXSN-*cyclin E* vector sequences.

Primary cultures of human fibroblasts and rat Rat-1 cells were infected with LXSN-*cyclin E* vector particles, or as a control with LXSN (as described in Example 9). The transduced cells were tested for expression of *cyclin E* (as described above), and LXSN-*cyclin E*-transduced Rat-1 and human fibroblasts were found to express 3- to 5-fold greater levels of cyclin E protein than the cells from which they were derived (and 3- to 5-fold greater than control LXSN-transduced cells). The growth-factor dependence of LXSN-*cyclin E*-transduced human cells was determined by measuring tritiated thymidine incorporation into DNA in serum free medium (D-MEM) or medium supplemented with 10%, 1%, 0.1%, or 0.01% (v/v) fetal bovine serum (Table 2) and the growth factor dependence of Rat-1 cells was determined by measuring BrdU incorporation in 10%, 1.0% or 0.1% serum (FIGURE 18A-18B). In the BrdU assay, only cells that are synthesizing DNA (i.e., S-phase cells) incorporate BrdU into DNA and score positive in the assay. Therefore, the rate of accumulation of BrdU positive cells can be taken as a relative measure of the rate at which cells transition from the conclusion of one mitosis through G1-phase and into the next round of DNA synthesis (i.e., S-phase). The results presented in FIGURES 18A and 18B show the percent of the total cell nuclei that were labeled with BrdU in cultures of LXSN-transduced control Rat-1 cells ("RAT1/LX", open circles) and LXSN-*cyclin E*-transduced cells ("RAT1/cyclin E", closed circles) as a function of time after releasing mitotic arrest induced by nocodazole treatment. The growth factor dependence of the cells was evaluated by culturing the cells in 10%

bovine calf serum (FIGURE 18A) or in 1% or 0.1% serum (FIGURE 18B). The results in FIGURES 18A and 18B show that a) irrespective of the percentage of serum in the culture medium, the LXSN-*cyclin E*-transduced cells initiated DNA synthesis more rapidly than control cells; and, b) the LXSN-*cyclin E*-transduced cells exhibited increased resistance to low serum (i.e., 0.1%) and initiated DNA synthesis about 10-12 hours sooner than the control cells (FIGURE 18B).

In a similar manner, the results presented in Table 2 show that LXSN-*cyclin E*-transduced human fibroblasts and Rat-1 cells exhibite reduced growth factor requirements for proliferation. In control cells (i.e., LXSN-transduced cells) when serum was reduced from 10% to 0.1% the cells continued to proliferate and incorporate thymidine into DNA, although at a reduced rate, with levels 11% of those observed in the presence of optimal levels of growth factors (i.e., 10% serum). In contrast, the growth of LXSN-*cyclin E*-transduced cells was reduced to 19% of maximal levels (seen in 10% serum) but this level was more than 2-fold higher than the level observed in the control LXSN-transduced cells. In addition, when PDGF (10ng/ml) was added to *cyclin E*-transduced cells growing in 0.1% serum, the levels of proliferation were 50% of the maximal level occuring in the presence of 10% serum (Table 2). In contrast, when PDGF was added to control human fibroblasts growing in 0.1% serum no stimulation of thymidine incorporation was observed.

Table 2

Growth Factor Dependence of Vector-Transduced Human Fibroblasts

Transducing Vector	Serum (%)	PDGF[a]	^{3}H-TdR (CPM)[b]	Percent Max CPM[c]
LXSN	10	0	524.300	100
	0.1	0	59.359	11
	0.1	+	55.788	11
LXSN-*cyclin E*	10	0	708.871	100
	0.1	0	137.712	19
	0.1	+	356.284	50

a.) + = PDGF (10 ng/ml) added to the culture media; 0 = no PDGF;
b.) ^{3}H-TdR, tritited thymidine incorporation determined 36 hours after adding 1-2 μCi/ml ^{3}H-TdR to culture medium;
c.) Percent Max CPM, % maximal ^{3}H-TdR CPM = (CPM in 0.1%)/(CPM in 10% serum)

(Flow cytometric analysis confirmed the continued presence of cycling cells in LXSN-*cyclin E* transduced Rat-1 and human fibroblast cells in the presence of 0.1% serum.). The combined results show that the LXSN-*cyclin E*-transduced Rat-1 cells have a reduced growth factor dependence for transitioning the G1 phase of the cell cycle.

These combined results support the hypothesis that over-expression by 3-5 fold of a single cyclin, i.e., *cyclin E*, can *partially* (but not completely) restore the ability of cells to proliferate in the absence of growth factors, and *fully* restore proliferation in the presence of a single growth factor, PDGF. Thus, the results tend to favor a unitary hypothesis in which one cyclin and one growth factor regulate growth of a cell at a particular stage of differentiation; however, this interpretation is not supported by the quantitative aspects of the data, i.e., over-expression did not render the cells *completely* growth factor independent. Therefore, the possibility also exists that cyclins other than cyclin E are participating in the stimulation of cell proliferation in the presence of PDGF. (The results could thus be interpreted as providing support for a multiform model of cell proliferation where activity of several cyclins and growth factors combines to promote cell proliferation.)

In summary, the results can be interpreted to provide support for either a unitary or multiform model of cell proliferation. Aside from any interpretations, the results are significant for demonstrating that genetic manipulation of a single cyclin in a cell and treatment with a single growth factor is sufficient to dramatically alter the conditions required to grow cell *in vitro*. It appears from the results that with the proper combination of G1 cyclin expression in a particular cell: a) a cell line may be produced whose proliferation is largely unconstrained in the absence of exogenous growth factors; and b) a cell line may be produced whose proliferation is largely dependent upon one or more selected growth factors. In viewing the potential long-term significance of the present findings it may be worthwhile to recall that it took Sam Hanks nearly 10 years of research to develop Hank's Balanced Salt Solution (HBSS); about an additional 3-5 years for Eagle to develop Eagle's Minimal Essential Medium (MEM); and still longer for Rene Dulbecco to achieve a D-MEM formulation. (Media such as RPMI 1640 and M199 still carry a number that designates how many formulations preceeded their development.) The findings described herein are thus highly significant, for showing that simple manipulation of a single protein in a cell is sufficient to promote propagation of the cell *in vitro* in the near complete absence of serum growth factors.

Materials and Methods

Plasmids and libraries: The human cDNA library was a gift from J. Colicelli and M. Wigler. It was prepared from the human glioblastoma cell line U118 in the vector *pADNS* (Colicelli et al., 1988). The portion of the library used in these experiments contained cDNA inserts that had been selected to be >2 kb. In the experiments involving isolation of human *CDC2* homologs, the *cyclin E* cDNA was

transferred to the vector *pMAC*. This 2μ-based vector uses the *ADH* promoter to drive expression of the human cDNA and contains the *TRP1* selectable marker. For expression of *cyclin E* in *E. coli* a SmaI-PvuII fragment containing the entire *cyclin E* coding region was cloned into the unique SmaI site in the vector *pGEX-3T* (Amgen). For *in vitro* transcription/translation reactions, the SmaI-NotI fragment of *cyclin E* was cloned into the MscI site in the vector *pCITE-1* (Novagen). For *in vitro* translation of human *cyclins A* and *B*, cDNAs with genetically engineered NcoI sites at the initiating methionine were generously provided by Jonathan Pines and Tony Hunter. *PCITE* vector was cleaved with SalI, blunt-ended with Klenow enzyme and then cleaved at the unique NcoI site. *Cyclin* cDNAs were isolated by cleavage with EcoRI (for *cyclin A*) or BamHI (for *cyclin B*), blunt-ended with Klenow and then cleaved with NcoI. The *Xenopus CDK2* clone, *pEMBLYe30/2*, has been described previously (Paris et al., 1991). For some assays in yeast, the *cyclin A*, *B*, and *E* cDNAs were subcloned into the vector *pADANS*, which is identical to *pADNS* except that the first 10 amino acids of the ADH protein are fused to the expressed protein.

Antibodies: The peptide YLDNQIKKM (SEQ. ID. NO. 3), corresponding to the C terminus of human CDC2, was synthesized chemically and covalently coupled to BSA via the tyrosine residue for injection into rabbits. For affinity purification, rabbit serum was precipitated with 50% ammonium sulfate, resuspended in 10 mM sodium phosphate (pH 8.0) and dialyzed extensively with 10 mM sodium phosphate, 0.15M NaCl, pH7.2 (PBS). Affinity columns were prepared by coupling the peptide to CNBr-activated Sepharose using conditions recommended by Pharmacia. The dialysate was applied to the affinity column equilibrated in PBS. The follow-through was subsequently reloaded twice. The column was washed with 10 column volumes of PBS + 2 M KCl, and protein subsequently was eluted with 10 column volumes of 5 M NaI + 1 mM sodium thiosulfate (made fresh before use). Fractions containing immunoglobulin were determined by absorbence at 290, pooled, and dialyzed extensively against PBS. The peptide CEGVPSTAIREISLLKE (SEQ. ID. NO. 4), corresponding to the conserved "PSTAIRE" domain of the *CDC2* gene family, was synthesized chemically and coupled to keyhole limpet hemocyanin (KLH) via the cysteine residue, and antibodies were prepared in rabbits and affinity purified as described above. The peptide YDEAEKEAQKKPAESQKIERE (SEQ. ID. NO. 5), corresponding to residues 104-123 of human cyclin A, was synthesized chemically and coupled to BSA, and antibodies were prepared in rabbits and affinity purified as described above.

Antibodies directed against CDK2 were raised against a peptide corresponding to the 15 C-terminal amino acids of human CDK2 coupled to keyhole limpet hemocyanin. Two other antisera against the 9-C-terminal amino acids of human CDK2 were also used in the course of these experiments (Elledge et al., 1992; Rosenblatt et al., 1992). The polyclonal anti-cyclin E antisera has been described (Koff et al., 1991).

For preparation of cyclin E antibodies, *E. coli* containing *GEX-cycE* (see below) were grown to an OD_{600} of 0.4-0.6, and fusion protein expression was induced with 10 mM IPTG. After 3 hours of additional growth at 30°C, the *E. coli* were pelleted, washed once with PBS, and again with GEX buffer A (60 mM Tris-HCl pH 8.0, 25% sucrose, 10 mM EDTA) and stored at -75°C. Cells were resuspended in 1/30 the original culture volume in GEX buffer A containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor (SBTI), and 10 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). Protease inhibitors were used in all subsequent steps. SDS was added to 0.03% and cells were lysed by sonication. Lysates were clarified by centrifugation at 13,000 x g and added to a 1:1 slurry of Sepharose CL4B in GEX buffer C (0.02 M HEPES-KOH (pH 7.6), 100 mM KCl, 1.2 mM EDTA, 20% glycerol, and 1 mM DTT) with 0.03% SDS, incubated for one hour at 4°C, and the Sepharose removed by low speed centrifugation. Cleared lysates were incubated with glutathione-agarose beads (SIGMA #G4510) (approximately 360 μg of GEX-cyclin E per ml of glutathione-agarose beads) for 1 hour at 4°C. The agarose beads were pelleted and washed 5 times with 10 volumes of GEX buffer C with 0.03% SDS, and the cyclin E fusion protein (GEX-E) eluted with buffer C with 0.03% SDS plus 5 mM glutathione. Fractions containing GEX-E were identified by SDS-PAGE electrophoresis and Coomassie blue staining. Rabbits were injected with 400 μg of total GEX-E protein in complete Freund's adjuvant; 320 μg was injected subcutaneously and 80 μg intramuscularly. Rabbits were boosted every 3 weeks with an identical regimen except incomplete Freund's adjuvant was used. Bleeds were obtained 7 days postinjection and analyzed by their ability to immunoprecipitate cyclin E produced in a rabbit reticulocyte lysate (Promega).

The specificity of the cyclin E antiserum was demonstrated by immunoprecipitation of *in vitro* translated cyclin E, A, and B. *In vitro* translated cyclins were made according to manufacturer's directions. Briefly, plasmids were linearized with either NheI (*cyclin B/cyclin E*) or PstI (*cyclin A*). *Cyclin A* was subsequently blunt ended with the Klenow enzyme before the transcription reaction.

Transcription was carried out using the T7 RNA polymerase, and RNA was isolated by ethanol precipitation. Rabbit reticulocyte lysates were programmed with the RNA and incubated for 2 hours at 30°C. Programmed lysate (5 μl) was incubated with 10 μl of cyclin E antisera in 500 μl of 50 mM Tris-HCl pH 7.4, 250 mM NaCl, and 0.1% NP-40 for 1 hour at 4°C. Protein A-Sepharose was added and incubation continued for 1 hour. Protein A beads were pelleted and washed 4 times with 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/ml BSA. The immunoprecipitates were resuspended in sample buffer and run on 12% SDS-PAGE gels. The gels were fixed and enhanced with 1 M sodium salicylate before drying and autoradiography.

Cyclin E antibodies were affinity purified on columns of GST-cyclin E fusion protein. Approximately 100 ml of rabbit sera was precipitated with 50% ammonium sulfate. The precipitate was collected at 8,000 X g and resuspended in 10 mM sodium phosphate pH 8.0 and dialyzed against PBS. The dialysate was adjusted to 10% glycerol and pre-cleared over a glutathione-S-transferase (GST) column. Flow through fractions were collected and the column regenerated by washing with 0.2 M glycine pH 2.2. The column was re-equilibrated with PBS and this process was repeated 3 times.

Cleared sera was subsequently applied to a GST-cyclin E column. Following adsorption, the column was washed first with PBS and then with 2 M KCl-PBS, and bound antibody was eluted with NaI-sodium thiosulfate as described (Koff et al., 1991). The eluate was dialyzed against coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) and concentrated 5 to 10-fold using Centricon 10 concentrators (Amicon).

<u>DNA Sequencing</u>: Nested deletions of the *cyclin E* cDNA were sequenced on both strands using dideoxy chain termination methods.

<u>Kinase assays</u>: GEX-cyclin E (GEX-E) was purified as described up to the washing of the GEX-E bound to glutathione-agarose. For this experiment the beads were washed 3 times with 5 volumes of GEX buffer C with 0.03% SDS, 5 times with 10 volumes of buffer C with 0.5% Triton X-100, 5 times with 10 volumes of buffer D (30 mM HEPES-KOH pH 7.6, 7 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT). 100 μl of GT-cyclin E-Sepharose beads, p13-Sepharose (5 mg of p13 per ml Sepharose), GT-Sepharose, or blank Sepharose were incubated with 100 μg of S-100 extract from human MANCA G1 cells (Roberts & D'Urso, 1988) in conditions used for *in vitro* replication of SV40 DNA (buffer D plus 3 μg creatine phosphokinase, 40 mM phosphocreatine, 0.25 mM dNTPs, 0.5 mM CTP, UTP, and GTP, 3 mM ATP). The

beads were then pelleted and washed 5 times in kinase buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT) plus 0.1 mg/ml BSA. For kinase assays the beads were resuspended in 50 µl kinase buffer + 30 µM ATP, 5 µCi γ-^{32}P-ATP, and 1 µg histone H1, and incubated at 37°C for 30 minutes. Products were analyzed by SDS-PAGE followed by autoradiography.

For studying the kinase bound to the SDS-GT-cyclin E-Sepharose beads, the GT-cyclin E beads and GT beads were prepared and incubated with G1 extracts and washed as described. Incubation of the beads at 37°C for 30 minutes in TNT (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and 5 mM glutathione (reduced form) was sufficient to release the proteins bound to the beads by interaction with glutathione. The supernatant was transferred to a fresh tube and immunoprecipitated with affinity-purified antisera directed against the C-terminus of p34 cdc2. The immunoprecipitates (with Protein A-Sepharose) were washed three times in TNT and used in a histone H1 kinase assay as described.

To show phosphorylation of the GT-cyclin E protein by the bound CDC2 kinase, GT-cyclin E beads were prepared and incubated with G1 extracts and used in a kinase assay as described previously for histone H1; however histone H1 was not included in the assay. After incubation the pellet is washed with H1 kinase buffer + 0.1 mg/ml BSA, then with 30 mM HEPES-KOH pH 7.5, 7 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 0.2 M NaCl, and finally with TNT. The beads were then incubated with 1 ml of TNT and 5 mM glutathione (pH 7.5) at 37°C for 30 minutes to release the GT-cyclin E fusion protein. The supernatant was then collected and immunoprecipitated with antisera directed against cyclin E. Immune complexes were subsequently collected by adherence to Protein A-Sepharose. Immunoprecipitates were washed 3 times with TNT and products analyzed on 12% SDS-PAGE gels followed by autoradiography.

For immunoprecipitation of cyclin E from HeLa cell extracts, 2×10^6 cells were lysed in 50 mM Tris-HCl pH 7.4, 250 mM NaCl, and 0.1% NP-40 and clarified by ultracentrifugation at 100,000 x g for 30 minutes. Samples were immunoprecipitated using Protein A-sepharose with 15 µl of normal rabbit sera or sera generated against the cyclin E fusion protein. Immunoprecipitates were washed with kinase buffer and 0.1 mg/ml BSA, and the kinase assay was performed as described above.

T-peptide kinase assays were performed as described previously (D'Urso et al., 1990).

Yeast strains: Yeast strains used were isogenic with strain YH110 (Richardson et al., 1989). Unmarked deletions of *CLN1*, *CLN2*, and *CLN3* were constructed in this strain background. These deletions removed significant portions of the cyclin homology in *CLN1* and *CLN2* (Hadwiger et al., 1989; Cross & Tinklenberg, 1991) and completely deleted the *CLN3* coding sequence (Cross, 1990). All the deletion alleles were null alleles by the assays described previously (Richardson et al., 1989). These deletion alleles were unmarked, unlike the originally described *cln* disruptions (Richardson et al., 1989), and therefore were compatible with the plasmid transformation experiments performed here. The *cln*-deficient strain was kept alive by the *GAL-CLN3* plasmid described previously (Cross, 1990). The *cdc28-13* allele in this isogenic strain background was provided by D. Lew and was combined with the three *cln* deletions by mating and tetrad analysis.

Yeast transfections: Transfections were performed using the lithium acetate procedure according to the method of Schiestl and Gietz (1989). Yeast cells grown in galactose were transfected with 2 μg of library DNA in each of 50 independent aliquots. Transformants were selected on galactose for leucine prototropy and typically numbered 1000-2000 per plate. Colonies were grown for 2 days and then replica plated onto YEP-glucose. Colonies that grew on glucose were patched onto FOA medium (Boeke et al., 1984) to identify colonies that could grow without the *GAL-CLN3* plasmid. Plasmid DNA was rescued into *E. coli* by electroporation from colonies surviving this screen and minipreps were retransfected into 589-5 strain yeast. cells to confirm plasmid-dependent complementation of the triple *cln* deletion. For the screen identifying human *CDC2* homologs, colonies growing on glucose were tested for cosegregation of glucose growth and retention of the transfected plasmids.

Construction of cyclin E retroviral vector

The *cyclin E* retroviral vector (LXSN-*cyclin E*) was constructed by inserting a blunt-ended HindIII fragment of the human cyclin E cDNA HU4 (Koff et al., 1991) (which contains the entire open reading frame) into the HpaI site of LXSN, a murine retrovirus-based vector (Mill and Rosman, 1989), in the sense orientation.

Cells

MANCA cells were maintained at 2-5 x 10^5 cells/ml in RPMI plus 10% calf serum in an atmosphere containing 5% CO_2. Cells were fractionated from exponentially growing populations by centrifugal elutriation (Marraccino et al., 1992). For synchronization at the G1/S boundary approximately 1 x 10^8 G1 cells were collected from exponentially growing populations of MANCA cells by elutriation and inoculated into RPMI containing 10% calf serum and 5 μg/ml aphidicolin and allowed

to grow for 8 hours. Flow cytometric measurement of cellular DNA content was used to demonstrate the synchrony of the cell population. MANCA cells synchronized in G1 were prepared exactly as described previously (Marraccino et al., 1992).

Rat PC-12 cells were maintained in DMEM containing 5% fetal calf serum and 10% horse serum in an atmosphere containing 10% CO_2. To induce neuronal differentiation confluent cells were split 1:20 and on the second day the media was replaced with serum free medium. Cells were incubated in serum free media for 24 h and the medium was then changed to complete medium containing 50 ng/ml NGF. NGF is added every two days and cells were harvested after 4-5 days.

Rat 208F cells were maintained in DMEM plus 10% calf serum in an atmosphere containing 5% CO_2. To generate quiescent cells, the cells were washed twice with PBS and subsequently grown in DMEM with 0.1% calf serum for 48 hours.

To measure Gl length in Rat-1 cells, the cells were synchronized in pseudometaphase by the addition of nocodazole at 100ng/ml for 4 hours. The mitotic cells were collected by gentle pipetting. Cells were then rinsed with DMEM and plated at 2×10^4/35 mm dish with DMEM plus 10% bovine calf serum. Cells were pulsed labelled with tritiated thymidine (80 Ci/mmole; 2 μCi/ml) for 30 minutes at each time point. Incorporation of thymidine into DNA was measured as described (Roberts & D'Urso, 1988).

Rat-1 cells that constitutively expressed *cyclin E* were produced as described (Miller and Rosman, 1989). PA317 amphotropic retrovirus packaging cells were plated at 5×10^5 cells per 60 mm dish on day 1. On day 2, 1 μg of LXSN-*cyclin E*, or the control DNA LXSN, was transfected into cells using a modification of the calcium phosphate procedure (Ohtsubo et al., 1991). On day 3, the culture medium was replaced with fresh medium and PE501 ecotropic packaging cells were plated 10^5 cells per 60 mm dish. On day 4, PE501 cells were fed with 4 ml of fresh medium containing polybrene. Virus was harvested from the PA317 cells and 5 μl to 1 ml of this material were used to infest PE501 cells. On day 5 the PE501 cells were trypsinized and plated in 10 cm dishes in medium containing 0.8 mg/ml G-418. Dishes with small numbers of colonies were used for isolation of individual clones by using cloning rings. These clonal lines were then analyzed by Southern blot analysis and assayed for vector titer and suitable clonal lines containing unrearranged retroviral genomes propagated as virus-producing cell lines. The LXSN and LXSN-

cyclin E viruses were used to infect Rat-1 cells and G-418 resistant cell populations used for further studies.

Preparation of GST and GST-E columns

E. coli containing the plasmids *pGEX-2T* or *pGEX-2TcycE* (*GEN-cyclin E*) were grown to OD_{600}=0.4 and induced with 0.4 mM IPTG for 4 h at 30°C. Cells were harvested and washed once in PBS and stored at -70°C. GST encoded by *pGEX-2T* was prepared as described previously (Koff et al., 1991). Fusion protein GT-cyclinE (GT-cycE) encoded by *pGEX-2TcycE* was prepared using a modification of the method of Glotzer et al. (1991). The cell pellet from a 500 ml culture was sonicated in 7 ml of 10 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM $MgCl_2$, 5 mM DTT with protease inhibitors. The extract was clarified by centrifugation at 13,000 X g and the supernatant discarded. The pellet was resuspended in 7 ml TND buffer (0.2 M Tris-HCl pH 8.2, 0.5 M NaCl, 5 mM DTT) and pelleted again. After discarding the supernatant the pellet was resuspended in 8 M urea containing 5 mM DTT and mixed gently at 4°C for 4 hours. The resulting extract was clarified at 13,000 X g for 10 minutes and the supernatant dialyzed against TN buffer (i.e., TND buffer containing 1 mM DTT instead of 5 mM DTT).

At least 2.5 mg of either GT or GT-cycE were incubated with 1 ml of glutathione agarose beads for 2 hours at 4°C, and subsequently collected at 1000 X g and washed 3 times with TN buffer containing 1 mM DTT. Coupling of the GT or GT fusion protein to the glutathione agarose support was carried out using the following protocol. The support was transferred to a column and washed with 0.1 M borate buffer pH 8.0 followed by 0.2 M triethanolamine pH 8.2. Dimethylpimelimidate (DMP) cross linker (40 mM DMP, 0.2 M triethanolamine pH 8.2) was run into the column leaving just a meniscus. Coupling was continued for 1 hour at room temperature. After coupling, the column was moved to 4°C and washed with 40 mM ethanolamine pH 8.2, followed by 0.1 M borate buffer pH 8.0. To elute uncoupled protein, the column was washed with PBS containing 20 mM glutathione pH 7.5 and subsequently stored in PBS containing 0.5% azide.

H1 kinase assays

8.3×10^6 cells were lysed by sonication in 100 µl of H1 lysis buffer (50 mM Tris-HCl pH 7.4, 0.25 M NaCl, 0.5% NP40) containing protease inhibitors (1 mM PMSF, 20 µg/ml TPCK, 20 µg/ml SBTI, 10 µg/ml leupeptin). Sonicated lysates were clarified at 13,000 X g for 10 minutes at 4°C and the supernatant transferred to a fresh tube and diluted two-fold with fresh H1 lysis buffer.

50 µl of extract was immunoprecipitated with a 2 µl of polyclonal antisera against cyclin E, or the C-terminus of p34 CDC2 for 1 hour. For cyclin A immunoprecipitations, lysates were incubated with 5 µl of the C160 monoclonal antibody for 30 minutes and for an additional 30 minutes after addition of 2 µl of rabbit anti-mouse antibody. Immune complexes were collected on Protein A sepharose, washed 2 X with lysis buffer and 4 X with H1 kinase buffer (20 mM Tris-HCl pH 7.4, 7.5 mM $MgCl_2$, 1 mM DTT). H1 kinase reactions were performed as described previously (Koff et al., 1991).

Preparation of lysates for immunoprecipitation-Western blot analysis

Cells (8.3×10^6/100 µl) were lysed by sonication in SDS-RIPA (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 0.1 mM orthovanadate, 50 mM NaF) containing protease inhibitors. In these experiments, approximately 1 mg of affinity purified antibody, or 1 ml of cyclin E pre-immune sera was coupled to 1 ml of CNBr-activated sepharose according to the manufacturers recommendations. In the experiments using cells arrested at the G1/S boundary, immunoprecipitations were carried out with affinity purified antibodies coupled to CNBr-activated sepharose using 2.5×10^7 cells and 100 µl of antibody linked sepharose. For studies of cell cycle fractions obtained by centrifugal elutriation we used 1×10^7 cells with 30 µl of anti-cyclin E sepharose.

Immune complexes were allowed to form for 3 hours at 4°C and were then washed twice with SDS-RIPA containing 5 mg/ml BSA and 3 times with SDS-RIPA. Samples were suspended in Laemmli sample buffer and separated on 12% PAGE gels. Gels were transferred to nitrocellulose by semi-dry electroblotting and the membranes blocked with either 2% milk in TNT (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for CDC2 or CDK2, or 1% gelatin in TNT for cyclin E. Blots were probed overnight at room temperature with either a 1:300 dilution of affinity purified anti-CDC2, or 1:1000 dilution of anti CDK2 serum, or a 1:1000 dilution of affinity purified cyclin E antibody. Bound antibody was subsequently detected with ^{125}I-Protein A.

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While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated nucleic acid molecule capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 1 and 1185 of the human cyclin E cDNA sequence shown in FIGURE 2 or to its complementary strand.

2. The isolated nucleic acid molecule of claim 1, encoding a cyclin E polypeptide.

3. The isolated nucleic acid molecule of claim 2, wherein the cyclin E polypeptide is capable of binding and activating a cell division kinase.

4. The isolated nucleic acid molecule of claim 3, wherein the cell division kinase is selected from the group consisting of CDC2, CDC28, CDK2-XL, CDC2-HS, and CDK2-HS.

5. The isolated nucleic acid molecule of claim 2, wherein the cyclin E polypeptide is capable of shortening the G1 phase of the eukaryotic cell cycle.

6. The isolated nucleic acid molecule of claim 1, encoding a polypeptide capable of binding to an antibody that binds to the cyclin E polypeptide shown in FIGURE 2.

7. A recombinant expression vector comprising the isolated nucleic acid molecule of claim 1 operably linked to suitable control sequences.

8. Cells transfected or transduced with the recombinant expression vector of claim 7.

9. A method of producing a polypeptide capable of activating a cell division kinase shortening the G1 phase of the cell cycle, comprising culturing the cells of claim 8 to produce the polypeptide encoded by said isolated nucleic acid molecule.

10. A method of shortening the cell cycle in a mammalian cell, comprising transfecting or transducing the cell with the recombinant expression vector of claim 7.

11. A polypeptide encoded by the isolated nucleic acid molecule of claim 1.

12. An immunologic binding partner capable of specifically binding the polypeptide of claim 11.

13. An assay for detecting the level of a cyclin E in a biological fluid, comprising incubating the immunologic binding partner of claim 12 with said fluid under conditions suitable for forming a complex between the immunologic binding partner and the cyclin E, separating the complex from the free binding partner or the fluid, and detecting the cyclin E or the binding partner in the separated complex.

14. An assay for measuring the abundance of cyclin E:cell division kinase complexes in a biological fluid comprising separating the complexes from the fluid and assaying the separated complexes to determine the amount of the cyclin E or the cell division kinase.

15. A polypeptide recognized by an antibody capable of binding the human cyclin E amino acid sequence shown in FIGURE 2.

16. An antisense nucleotide sequence capable of binding to the cDNA of claim 1 or to an mRNA transcribed from the isolated nucleic acid molecule of claim 1 and inhibiting transcription of said cDNA or translation of said mRNA.

17. A method for lengthening the cell cycle in a mammalian cell, comprising transfecting or transducing the cell with the antisense nucleotide sequence of claim 16.

18. A polypeptide encoded by a nucleic acid capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 631 and 936 of the cyclin E cDNA hydrophobic alpha helix sequence shown in FIGURE 2.

19. A polypeptide encoded by a nucleic acid capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 385 and 645 of the cycline E cDNA conserved MRAIL sequence shown in FIGURE 2.

20. The polypeptide of claim 19, capable of binding a CDC protein kinase.

21. A polypeptide encoded by a nucleic acid capable of hybridizing under stringent conditions to a nucleotide sequence residing between positions 640 and 1185 of the cyclin E cDNA C-terminal sequence shown in FIGURE 2.

22. A polypeptide encoded by a nucleic acid capable of hybridizing under stringent conditions to a nucleotide sequence residing between positions 1048 and 1080 of the cyclin E cDNA C-terminal conserved sequence shown in FIGURE 2.

23. A transgenic yeast cell having a genome lacking *cln1*, *cln2*, and *cln3* and having an episomal CLN3-encoding nucleotide sequence operably linked to a selectable marker and control elements, the cell exhibiting a shortened G1 cell cycle phase when transformed with a mammalian cyclin nucleic acid.

24. A cell of yeast strain 589 5 (ATCC No. 74098) according to claim 23.

25. A method of cloning a mammalian cyclin nucleic acid molecule, comprising introducing a candidate nucleic acid molecule into the transgenic yeast cell of claim 23, and screening for whether the G1 phase of the cell cycle is shortened by at least 1 hour.

26. A transgenic yeast cell having a genome comprising a *cdc28-13* gene, a G1 *cln* gene, a mitotic *clb* cyclin gene, and a cyclin E gene, the cell exhibiting a shortened G1 cell cycle phase when the cell is transformed with a mammalian cell division kinase-encoding nucleic acid.

27. A method of cloning a mammalian *cdc* gene encoding a CDC protein capable of binding to and being activated by a cyclin E protein, comprising introducing a candidate nucleic acid into the transgenic yeast cell of claim 26 and screening for whether the G1 phase of the cell cycle is shortened by at least 1 hour.

28. A cell of the 1238-14C-cycE strain (ATCC No. 74099) according to claim 26.

29. A transgenic yeast cell having a genome lacking in *cln1*, *cln2*, and *cln3*, and having an episomal CLN3-encoding nucleotide sequence, and a mammalian cyclin nucleic acid sequence operably linked to a selectable marker and control elements, wherein said mammalian cyclin nucleic acid is capable of hybridizing under stringent conditions to the nucleotide sequence residing between positions 1 and 1185

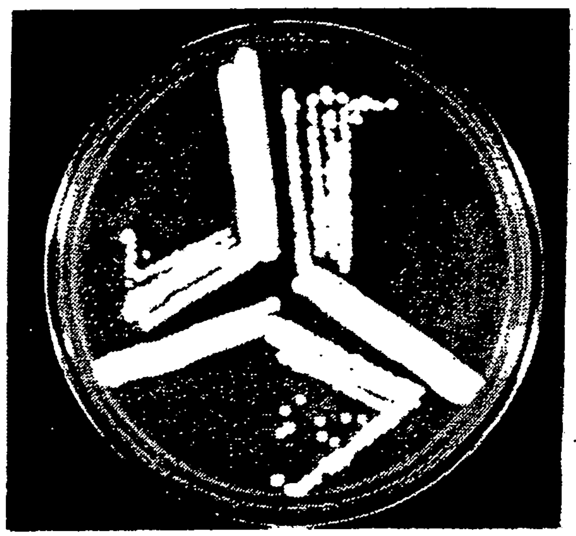
of the cyclin E cDNA sequence shown in FIGURE 2, and is capable of shortening the G1 phase of the cell cycle.

30. A method for cloning an inhibitor of a mammalian cyclin nucleic acid molecule, comprising introducing a candidate nucleic acid molecule into the transgenic yeast cell of claim 29, and screening for restoration of the duration of the G1 phase of the cell cycle.

31. The isolated nucleic acid molecule of claim 1, encoding a polypeptide having an activity that is greater or less than the activity of the cyclin E encoded by the cDNA sequence shown in FIGURE 2, wherein the activity is selected from among binding affinity of the polypeptide or cyclin E to a CDK2 kinase polypeptide, enzyme activity of the CDK2 kinase when the polypeptide or the cyclin E and the CDK2 are resident together in a polypeptide or cyclin E:CDK2 complex, and altered stability of the polypeptide:CDK2 complex as compared to the cyclin E:CDK2 complex.

GALACTOSE

589-5



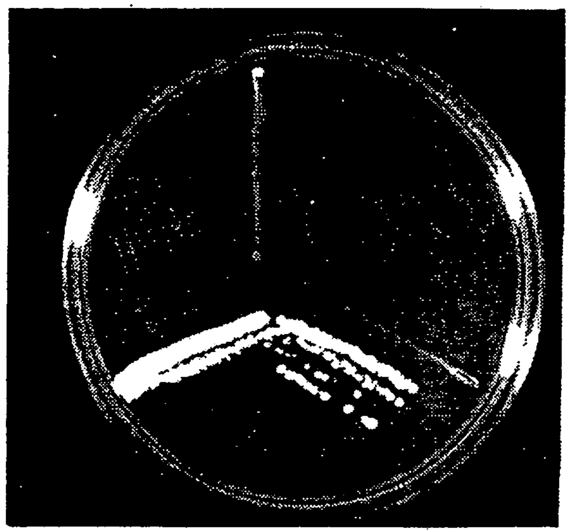
589-5
+pADNS

589-5
+pADNS-CYC E

Fig. 1A.

GLUCOSE

589-5



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+pADNS

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Fig. 1B.

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GTGCTCACCC GGCCCGGTGC CACCCGGGTC CACAGGGATG CGAAGGAGCG GGACACCATG   60
AAGGAGGACG GCGGCGCGGA GTTCTCGGCT CGCTCCAGGA AGAGGAAGGC AAACGTGACC  120
GTTTTTTTGC AGGATCCAGA TGAAGAAATG GCCAAAATCG ACAGGACGGC GAGGGACCAG  180
TGTGGGAGCC AGCCTTGGGA CAATAATGCA GTCTGTGCAG ACCCCTGCTC CCTGATCCCC  240
ACACCTGACA AAGAAGATGA TGACCGGGTT TACCCAAACT CAACGTGCAA GCCTCGGATT  300
ATTGCACCAT CCAGAGGCTC CCCGCTGCCT GTACTGAGCT GGGCAAATAG AGAGGAAGTC  360
TGGAAAATCA TGTTAAACAA GGAAAAGACA TACTTAAGGG ATCAGCACTT TCTTGAGCAA  420
CACCCTCTTC TGCAGCCAAA AATGCGAGCA ATTCTTCTGG ATTGGTTAAT GGAGGTGTGT  480
GAAGTCTATA AACTTCACAG GGAGACCTTT TACTTGGCAC AAGATTTCTT TGACCGGTAT  540
ATGGCGACAC AAGAAAATGT TGTAAAAACT CTTTTACAGC TTATTGGGAT TTCATCTTTA  600
TTTATTGCAG CCAAACTTGA GGAAATCTAT CCTCCAAAGT TGCACCAGTT TGCGTATGTG  660
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GCATATCTAA ATGACTTACA TGAAGTGCTA CTGCCGCAGT ATCCCCAGCA AATCTTTATA  840
CAGATTGCAG AGCTGTTGGA TCTCTGTGTC CTGGATGTTG ACTGCCTTGA ATTTCCTTAT  900
GGTATACTTG CTGCTTCGGC CTTGTATCAT TTCTCGTCAT CTGAATTGAT GCAAAAGGTT  960
TCAGGGTATC AGTGGTGCGA CATAGAGAAC TGTGTCAAGT GGATGGTTCC ATTTGCCATG 1020
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TAAGTGGGTC AAGTACACCA GCCACCTCCA GACACCAGTG CGTGCTCCCG ATGCTGCTAT 1500
GGAAGGTGCT ACTTGACCTA AAGGACTCCC ACAACAACAA AAGCTTGAAG CTGTGGAGGG 1560
CCACGGTGGC GTGGCTCTCC TCGCAGGTGT TCTGGGCTCC GTTGTACCAA GTGGAGCAGG 1620
TGGTTGCGGG CAAGCGTTGT GCAGAGCCCA TAGCCAGCTG GGCAGGGGGC TGCCCTCTCC 1680
```

Fig.2A.

Met Lys Glu Asp Gly Gly Ala Glu Phe Ser
Ala Arg Ser Arg Lys Arg Lys Ala Asn Val
Thr Val Phe Leu Gln Asp Pro Asp Glu Glu
Met Ala Lys Ile Asp Arg Thr Ala Arg Asp
Gln Cys Gly Ser Gln Pro Trp Asp Asn Asn
Ala Val Cys Ala Asp Pro Cys Ser Leu Ile
Pro Thr Pro Asp Lys Glu Asp Asp Asp Arg
Val Tyr Pro Asn Ser Thr Cys Lys Pro Arg
Ile Ile Ala Pro Ser Arg Gly Ser Pro Leu
Pro Val Leu Ser Trp Ala Asn Arg Glu Glu
Val Trp Lys Ile Met Leu Asn Lys Glu Lys
Thr Tyr Leu Arg Asp Gln His Phe Leu Glu
Gln His Pro Leu Leu Gln Pro Lys Met Arg
Ala Ile Leu Leu Asp Trp Leu Met Glu Val
Cys Glu Val Tyr Lys Leu His Arg Glu Thr
Phe Tyr Leu Ala Gln Asp Phe Phe Asp Arg
Tyr Met Ala Thr Gln Glu Asn Val Val Lys
Thr Leu Leu Gln Leu Ile Gly Ile Ser Ser
Leu Phe Ile Ala Ala Lys Leu Glu Glu Ile
Tyr Pro Pro Lys Leu His Gln Phe Ala Tyr
Val Thr Asp Gly Ala Cys Ser Gly Asp Glu

Fig. 2B.

Ile Leu Thr Met Glu Leu Met Ile Met Lys
Ala Leu Lys Trp Arg Leu Ser Pro Leu Thr
Ile Val Ser Trp Leu Asn Val Tyr Met Gln
Val Ala Tyr Leu Asn Asp Leu His Glu Val
Leu Leu Pro Gln Tyr Pro Gln Gln Ile Phe
Ile Gln Ile Ala Glu Leu Leu Asp Leu Cys
Val Leu Asp Val Asp Cys Leu Glu Phe Pro
Tyr Gly Ile Leu Ala Ala Ser Ala Leu Tyr
His Phe Ser Ser Ser Glu Leu Met Gln Lys
Val Ser Gly Tyr Gln Trp Cys Asp Ile Glu
Asn Cys Val Lys Trp Met Val Pro Phe Ala
Met Val Ile Arg Glu Thr Gly Ser Ser Lys
Leu Lys His Phe Arg Gly Val Ala Asp Glu
Asp Ala His Asn Ile Gln Thr His Arg Asp
Ser Leu Asp Leu Leu Asp Lys Ala Arg Ala
Lys Lys Ala Met Leu Ser Glu Gln Asn Arg
Ala Ser Pro Leu Pro Ser Gly Leu Leu Thr
Pro Pro Gln Ser Gly Lys Lys Gln Ser Ser
Gly Pro Glu Met Ala

Fig. 2C.

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```
CYCLIN E
CYCLIN A   MLGNSAPGPA TREAGSALLA LQQTALQEDQ ENINPEKAAP
CYCLIN B        MALRV TRNSKINAEN KAKINMAGAK RVPTAPAATS

CYCLIN E                                              MKED
CYCLIN A   VQQPRTRAAL AVLKSGNPRG LAQQQ RPKT RRVAPLKDLP VNDE
CYCLIN B   KPGLRPRTAL GDIGNKVSEQ LQAKMPMKKE AKPSATGKV   IDK

                                     E
CYCLIN E   GGAEFSARSR KRKA NVTVF LQDPDEEMAK ID RTAR DQ
CYCLIN A   HVTVPPWKAN SKQP AFTIH VDEAEKEAQK KPAESQKIER
CYCLIN B   KLPKPLEKVP MLVPVPVSEP VPEPEPEPEP EPVKEEKLSP

CYCLIN E   CGSQPWDNNA VCADPCSLIP TPDKEDDDRV YPNSTCKPRI IAPS
CYCLIN A   EDALAFNSAI SLPGPRKPLV PLDYPMDGSF ESPHTMDMSI VLED
CYCLIN B   EPILVDTASP SPMETSGCAP AEEQLC QAF SDVILAVNDV DAED

                                    E
CYCLIN E   RGSPLPVLSW ANREEV  WK IMLNKEKTYL RDQHFLEQHP
CYCLIN A   EKPVSVNENP DYHEDIHTYL R  EMEVKCK PKVGYMKKQP
CYCLIN B     GADPNLCS EYBKDIYAYL RQLEEEQAVR PK   YL LBR

               MRAIL   DWL  V       L  ET         DR
CYCLIN E   LLQPKMRAIL LDWLMEVCEV YKLHRETFYL AQDFFDRYM
CYCLIN A   DITNSMRAIL VDWLVEVGEE YKLQNETLHL AVNYIDRFL
CYCLIN B   EVTGNMRAIL IDWLVQVQMK FRLLQETMYM TVSIIDRFM
```

Fig. 3A.

```
                       V        LQL G        A K EE YP P     F    T
CYCLIN E    ATQENVVKTL LQLIGISSLF IAAKLEEIYP PKLHQFAYVT
CYCLIN A    SSM SVLRGK LQLVGTAAML LASKFEEIYP PEVAEFVYIT
CYCLIN B    QNN CVFKKM LQLVGVTAMF IASKYEEFYP PEIGDFAFVT

            D            ME     L   L          L
CYCLIN E    DGACSGDEIL TMELMIMKAL KWRLSPLTIV SWLNVYMQ
CYCLIN A    DDTYTKKQVL RMEHLVLKVL TFDLAAPTVN QFLTQYFL
CYCLIN B    DNTYTKHQIR QMEMKILRAL NFGLGRPLPL HFLRRASK

                        L
CYCLIN E    VAYLNDLHEV LLPQYPQQIF IQIAELLDLC VLDVDCLEFP
CYCLIN A    HQQPANCKVE SLAMFLGELS LIDADPY LK YLPSVIAGAA
CYCLIN B    IGEV KVEQH TLAKYLMELT MLDYDM   VH FPPSQIAAGA

               LA                     Y
CYCLIN E    YGILAASALY HFSSSELMQK VSGYQWCDIE NCVKWMVPFA
CYCLIN A    F HLALYTVT GQSWPESLIR KTGYTLESLK PCLMDLHQTY
CYCLIN B    F CLALKILD NGEWTPTLQH YLSYTEESLL PVMQHLAKNV

CYCLIN E    MVIRETGSSK LK HFRGVAD EDAHNIQTHR  DSLDLLDKA
CYCLIN A    LKAPQHAQQS IREDYKNSKY HGVSLLNPP   ETLNL
CYCLIN B    VMVNQGLTKH MTVKNKYATS KHAKISTLPQ LNSALVQDLA

CYCLIN E    RAKKAMLSEQ NRASPLPSGL LTPPQSGKKQ SSGPEMA
CYCLIN B    KAVAKV
```

Fig. 3B.

Plasmid	CDC28	cdc28-13
pADNS	$<10^{-4}$	$<10^{-4}$
pADANS	$<10^{-4}$	$<10^{-4}$
pADNS-CYC E	0.26	0.0034
pADANS-CYC E	1.0	0.035
pADANS-CYC B	0.23	0.3

Fig.4.

Plasmid	30	38
pMAC-CYC E	$<2x10^{-4}$	$<2x10^{-4}$
pADNS-CDC2-HS	$<2x10^{-4}$	$<2x10^{-4}$
pADNS-CDK2-HS	$<2x10^{-4}$	$<2x10^{-4}$
pMAC-CYC E + pADNS-CDC2-HS	0.56	0.24
pMAC-CYC E + pADNS-CDK2-HS	0.10	$<2x10^{-4}$

Fig.5.

	G1/S		G2/M	
	galactose	glucose	galactose	glucose
	CLN/cdc28-13	*CYCE/cdc28-13*	*CLB/cdc28-13*	*CLB/cdc28-13*
30°C	+	−	+	+
38°C	−	−	−	−

Fig.6.

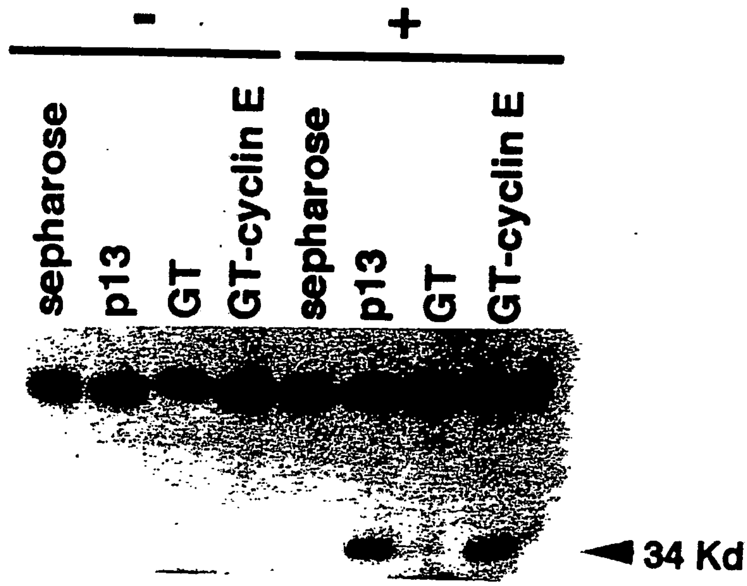


Fig. 7A.

Fig. 7C.

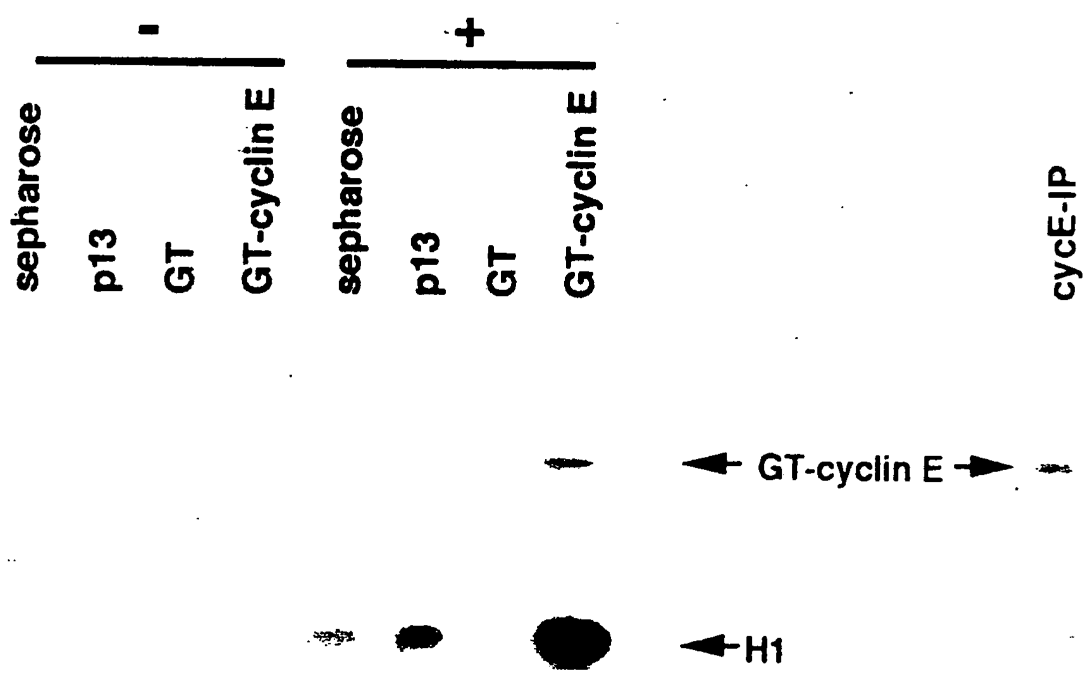


Fig. 7B.

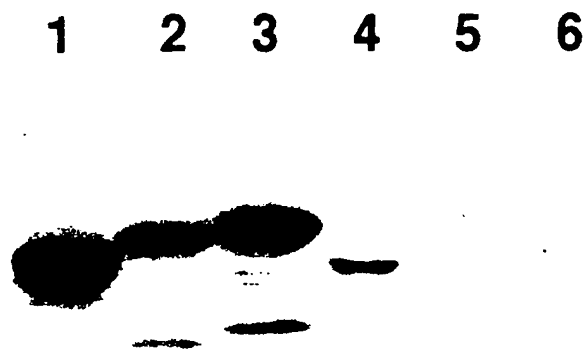


Fig. 8A.

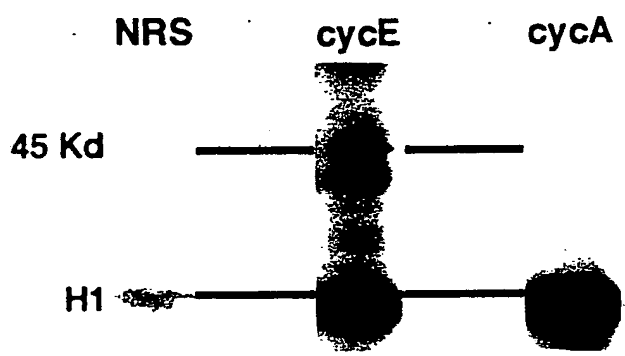


Fig. 8B.

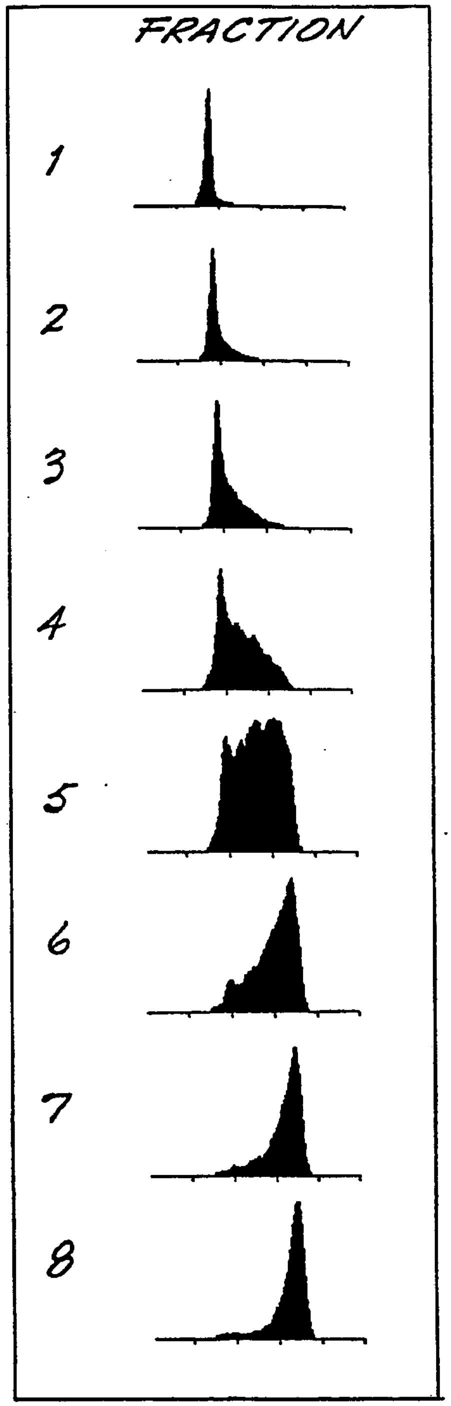


Fig. 9A.

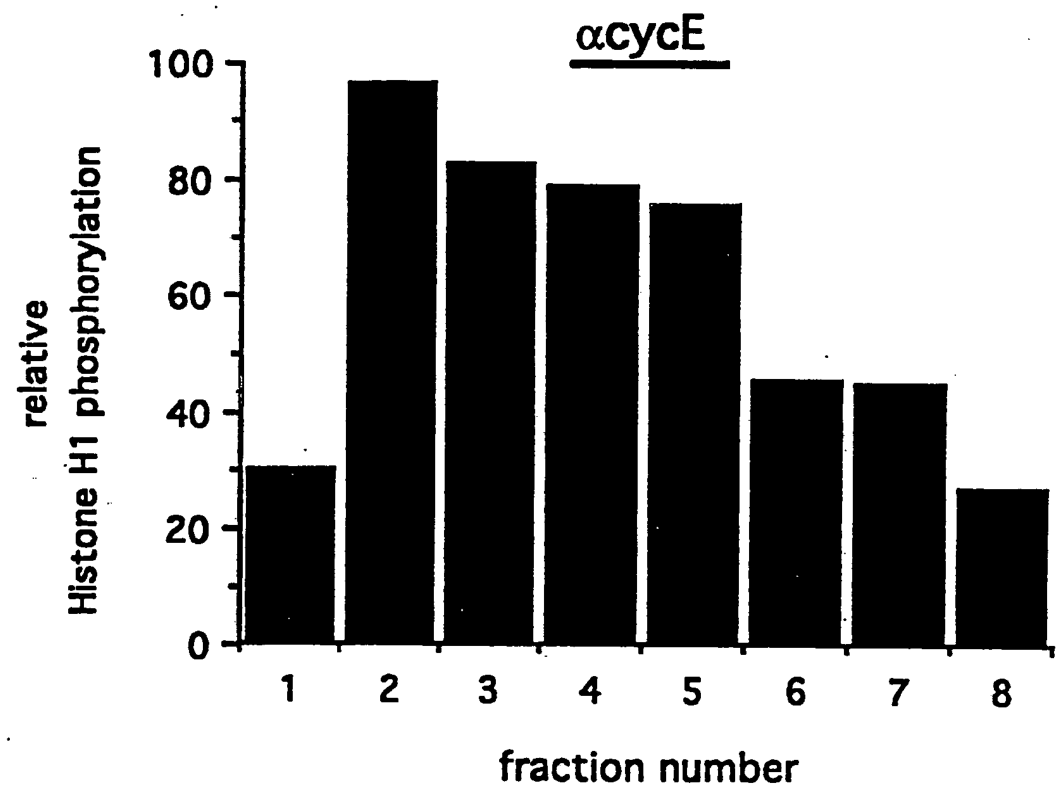

αcycE
relative
Histone H1 phosphorylation
100
80
60
40
20
0
1
2
3
4
5
6
7
8
fraction number

Fig. 9B.

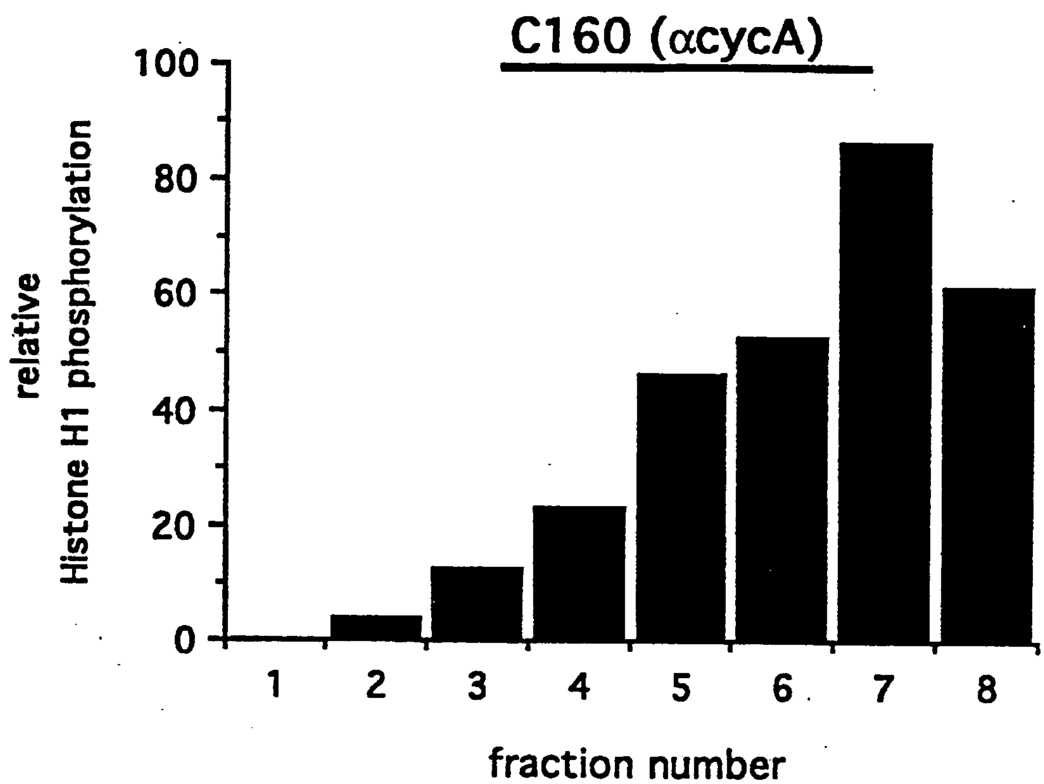

C160 (αcycA)
relative Histone H1 phosphorylation
100
80
60
40
20
0
1
2
3
4
5
6
7
8
fraction number

Fig. 9C.

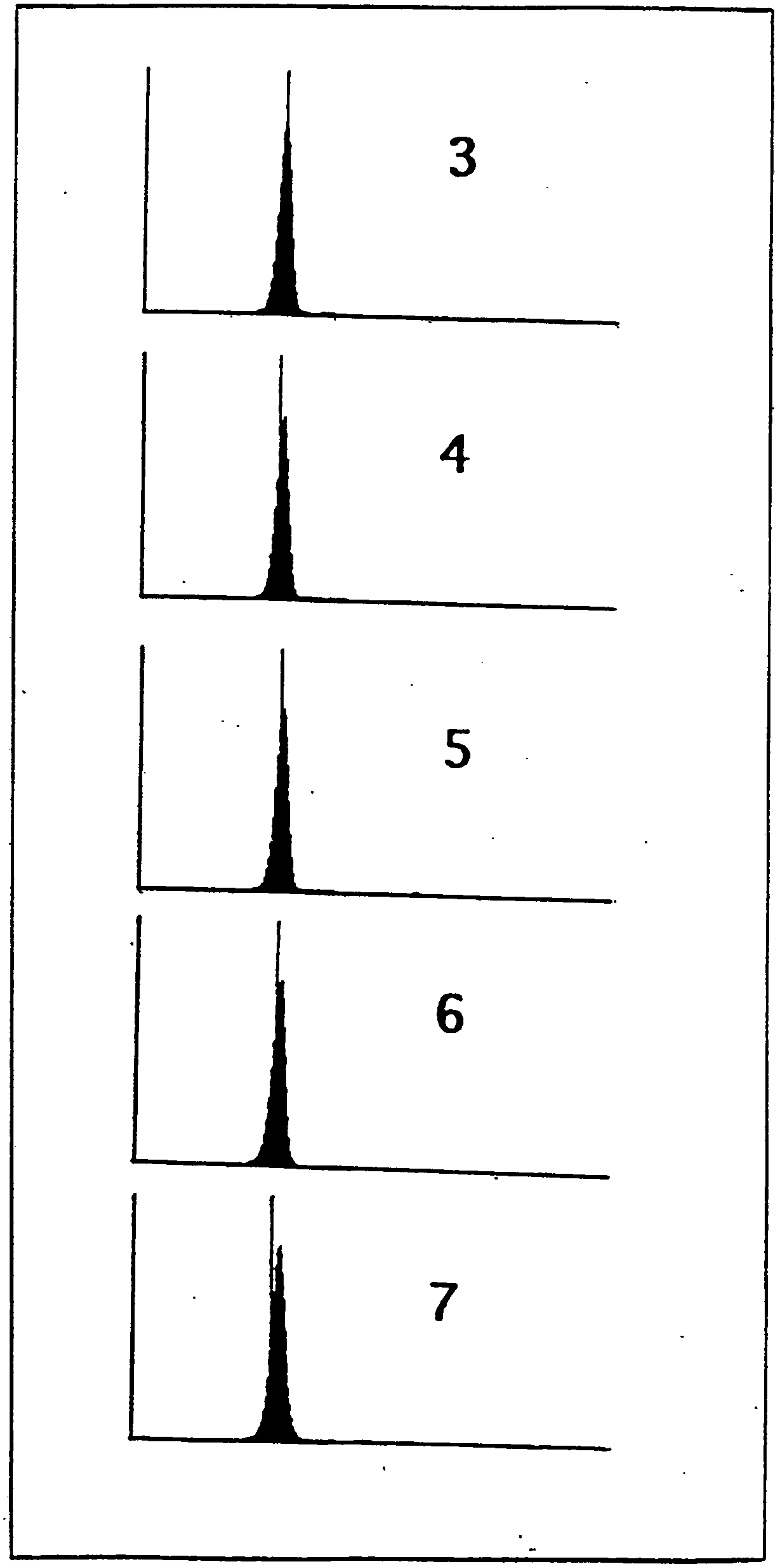


Fig. 9D.

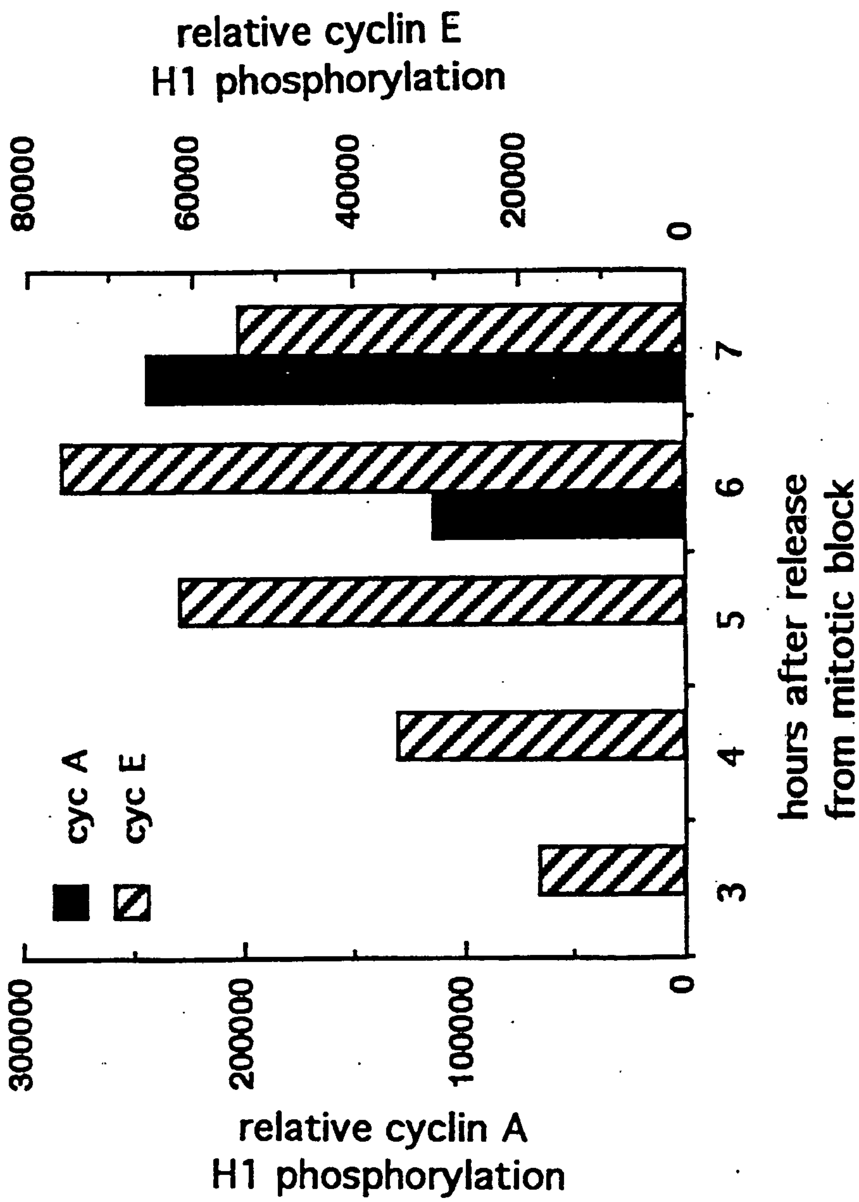

relative cyclin E
H1 phosphorylation
80000
60000
40000
20000
0
cyc A
cyc E
3
4
5
6
7
hours after release
from mitotic block
300000
200000
100000
0
relative cyclin A
H1 phosphorylation

Fig. 9E.

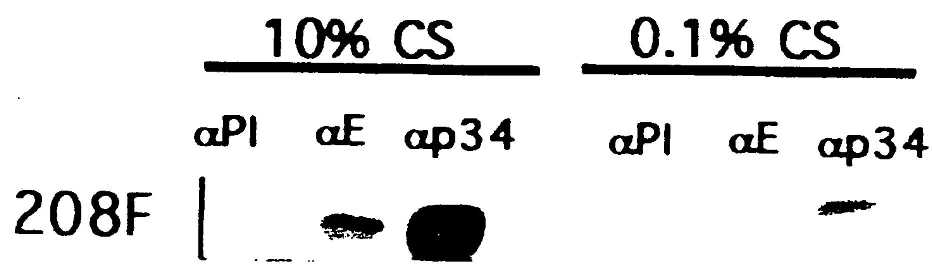


Fig. 10A.

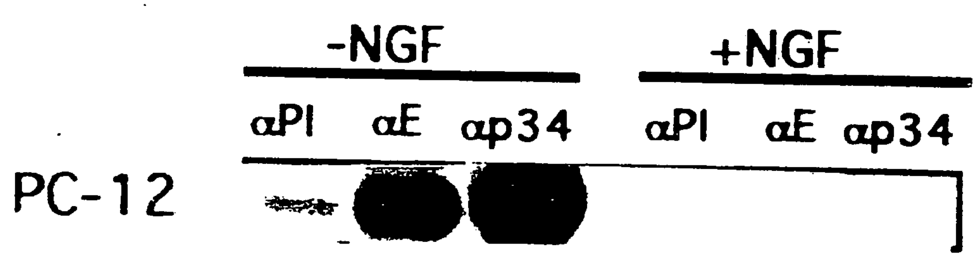


Fig. 10B.

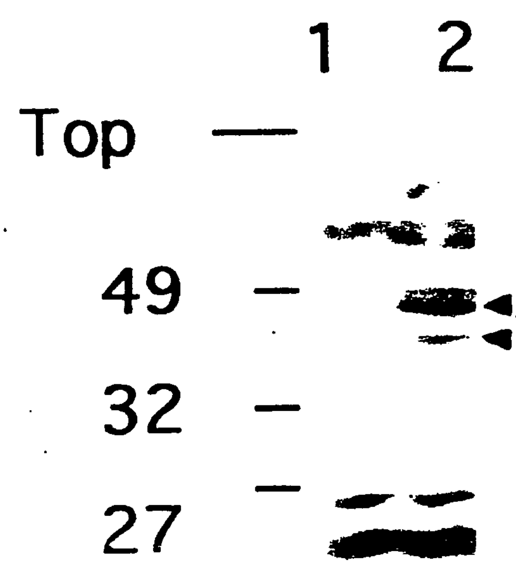


Fig. 11A.

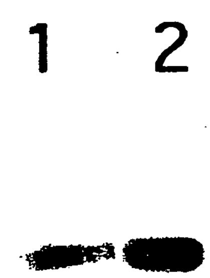
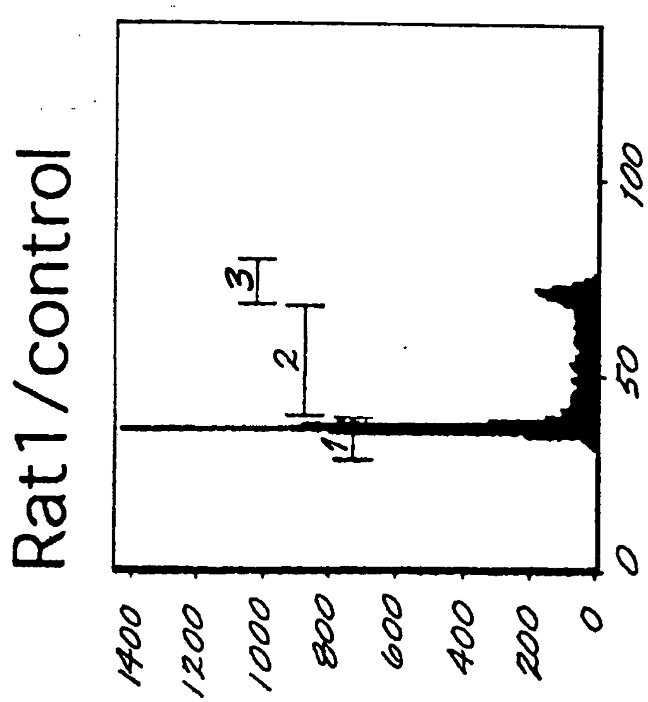
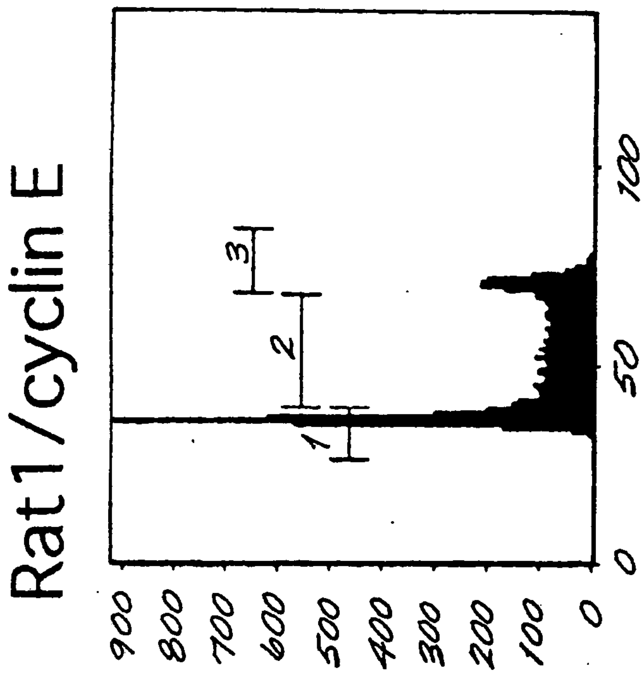


Fig. 11B.



G1: 55%
S: 31%
G2/M: 14%

Fig. 11C.



G1: 42%
S: 42%
G2/M: 16%

Fig. 11D.

20/27

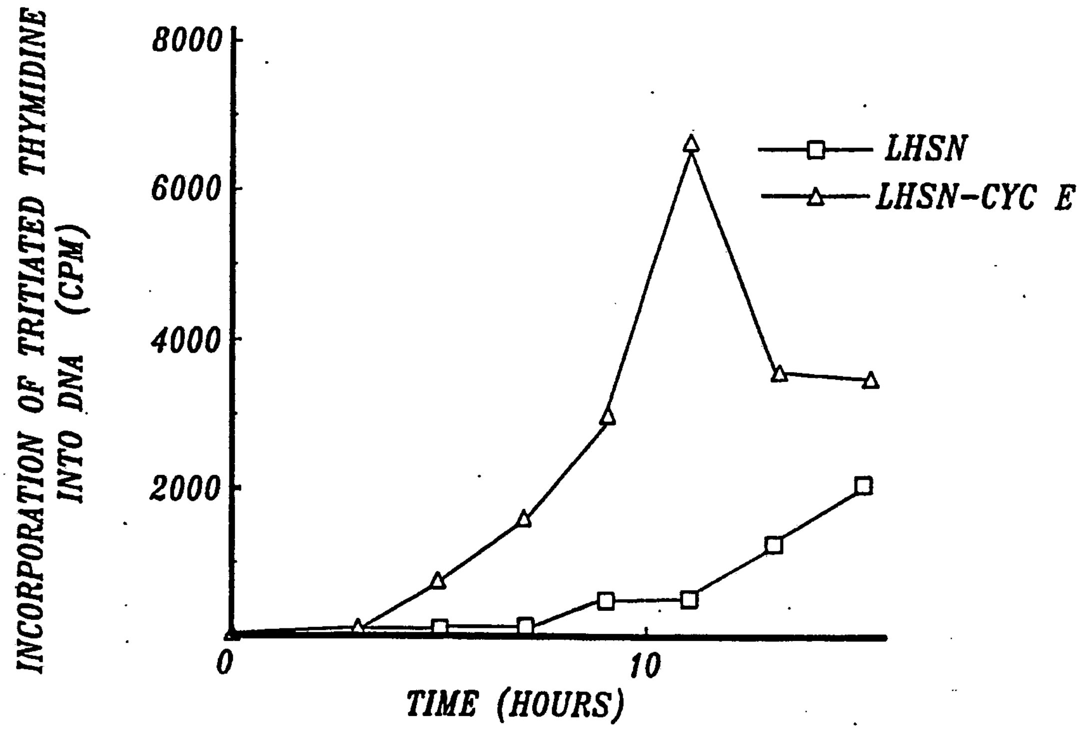


Fig.11E.

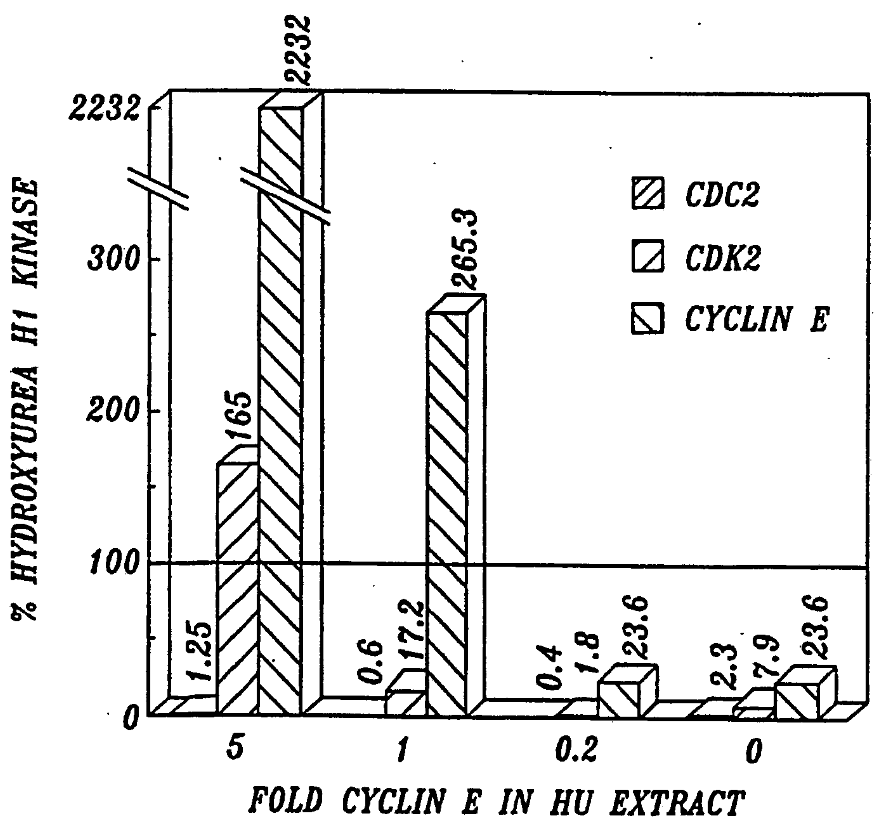


Fig.17.

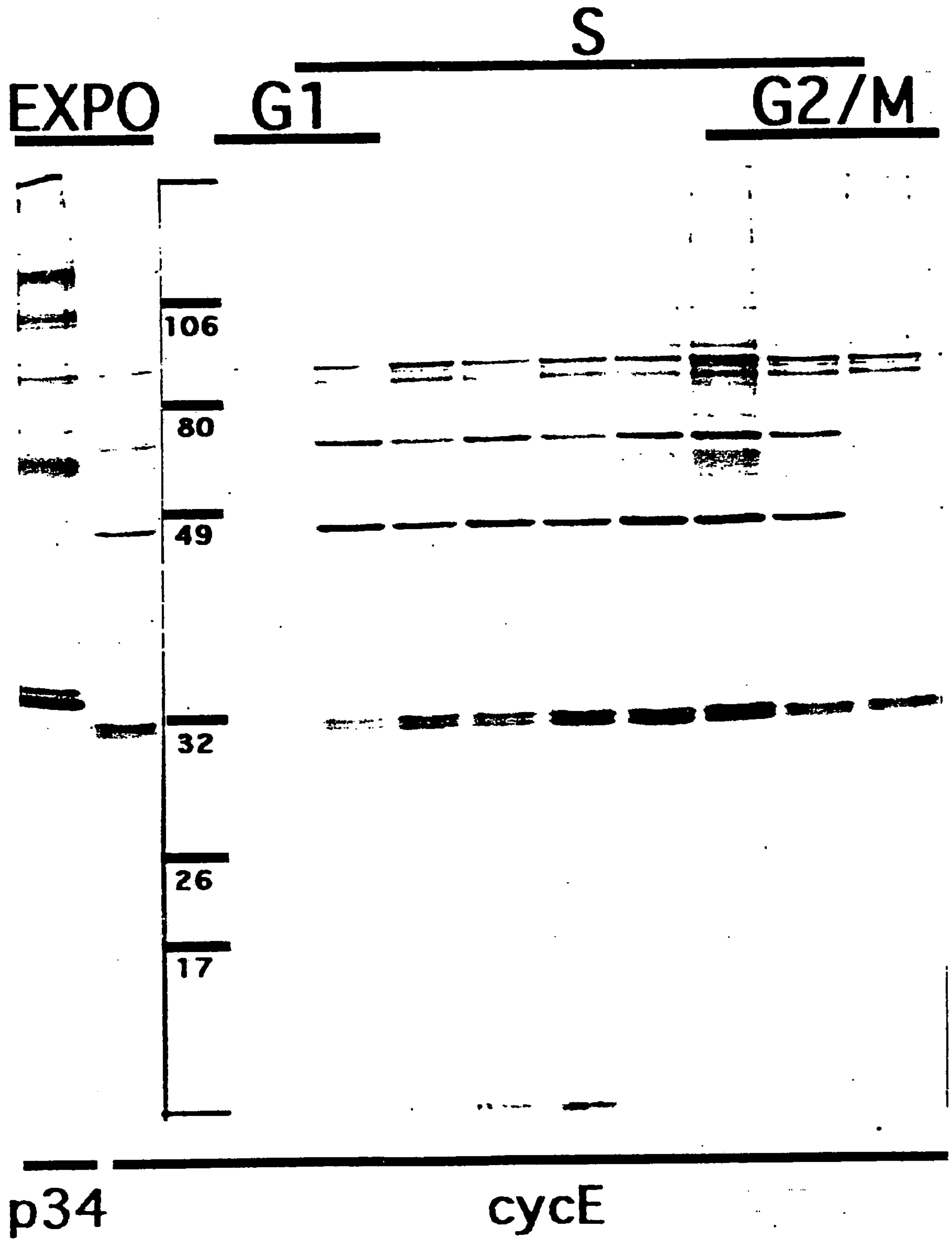


Fig. 12.

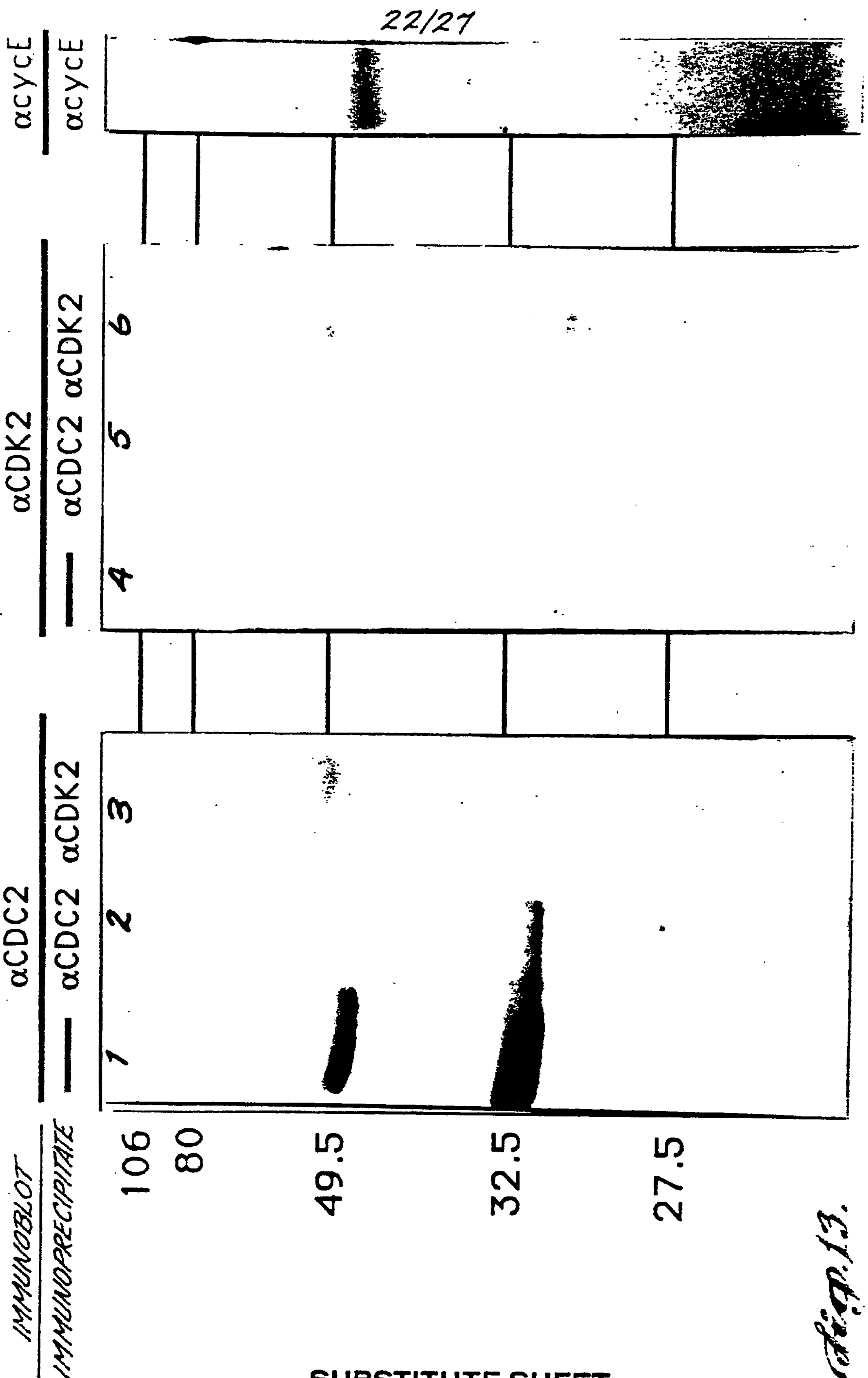

IMMUNOBLOT
IMMUNOPRECIPITATE
αCDC2
— αCDC2 αCDK2
αCDK2
— αCDC2 αCDK2
αcycE
αcycE
1
2
3
4
5
6
106
80
49.5
32.5
27.5

Fig. 13.

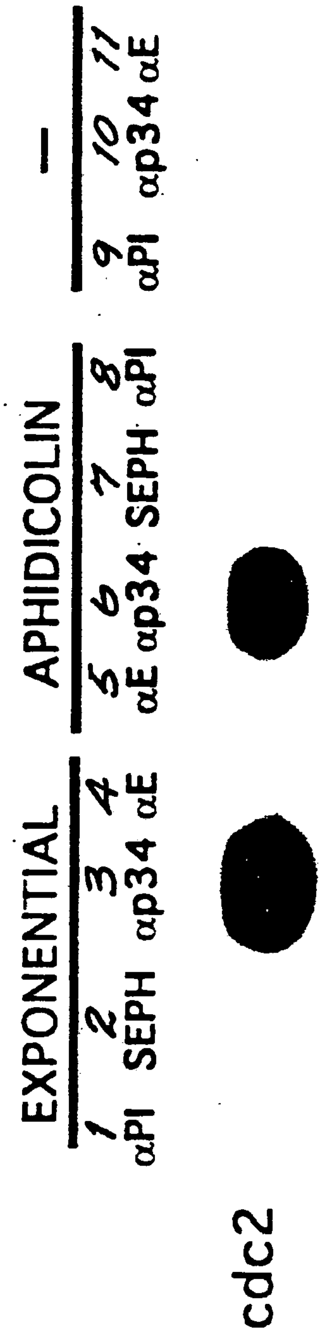


Fig. 14A.

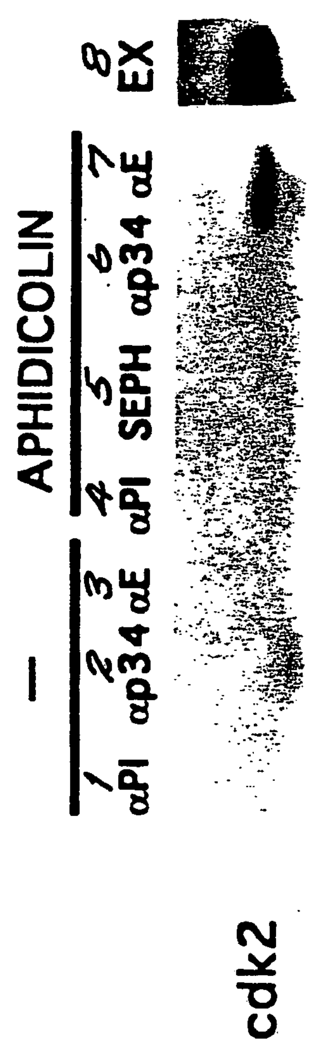


Fig. 14B.

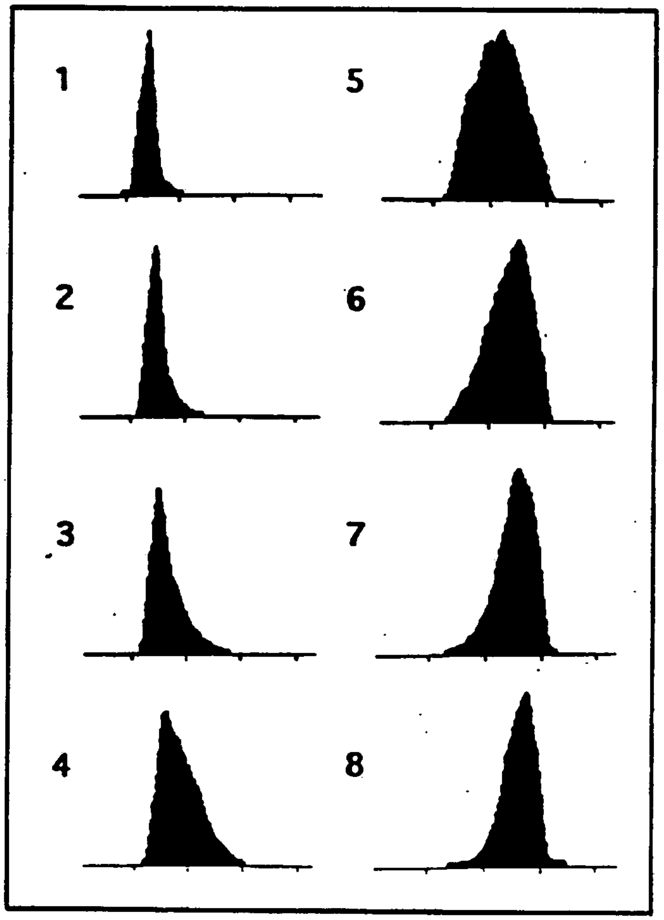


Fig. 15A.

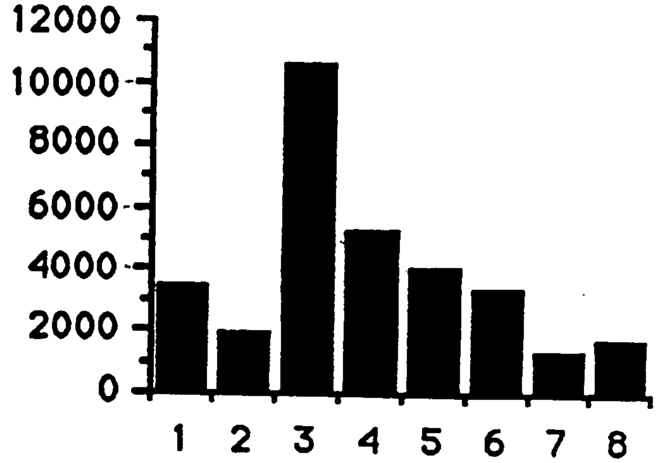
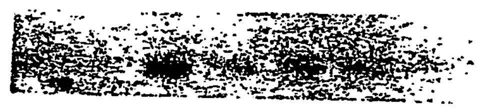


Fig. 15 B1.

Fig. 15 B2. cycE



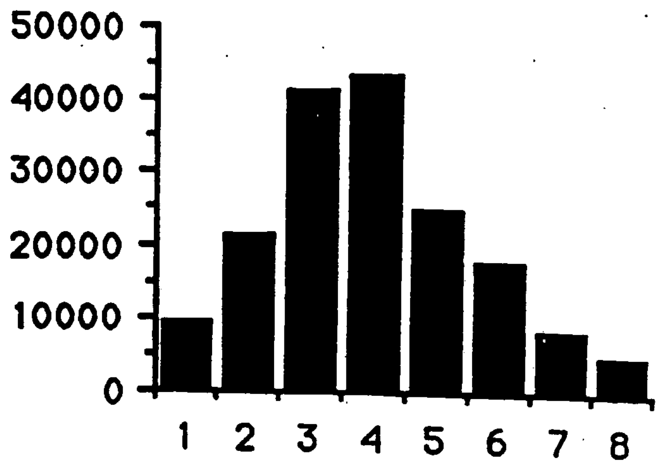


Fig. 15 B3.

Fig. 15 B4. cdk2

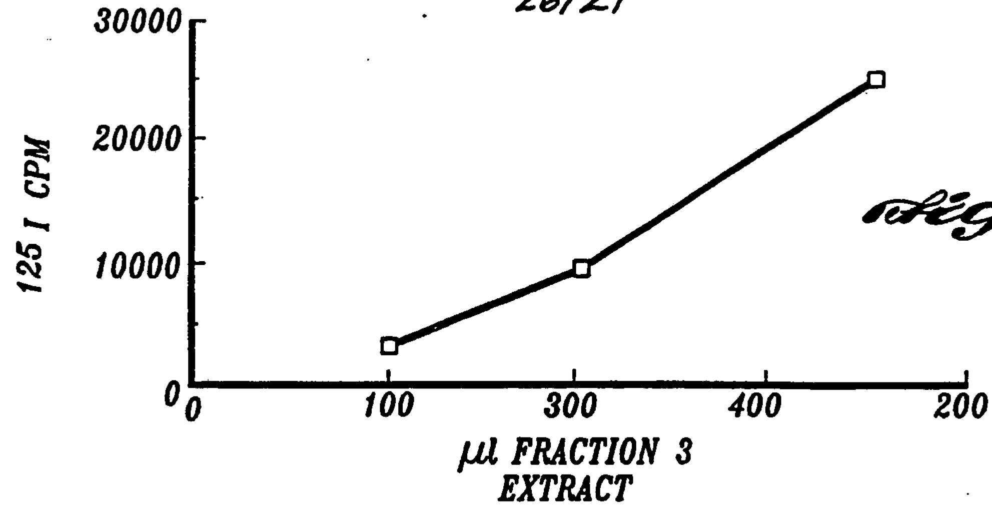


Fig. 15C.

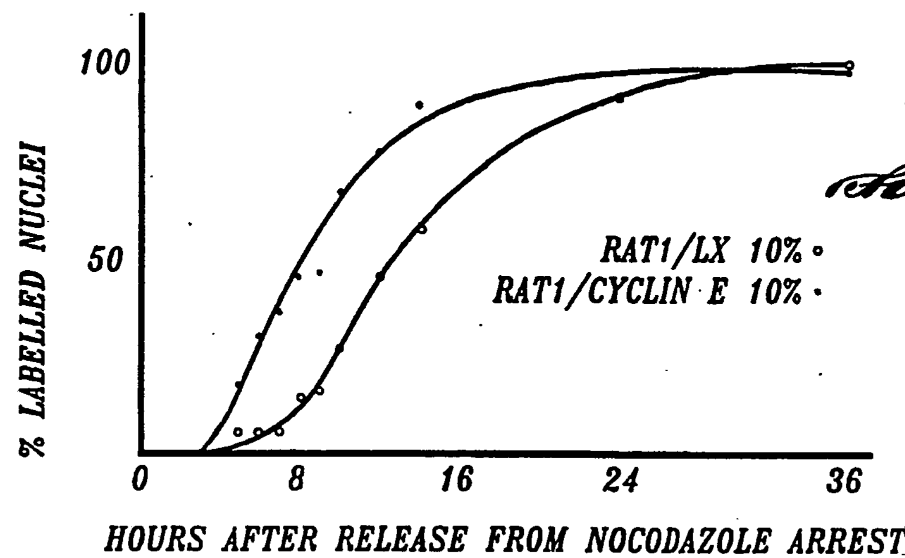


Fig. 18A.

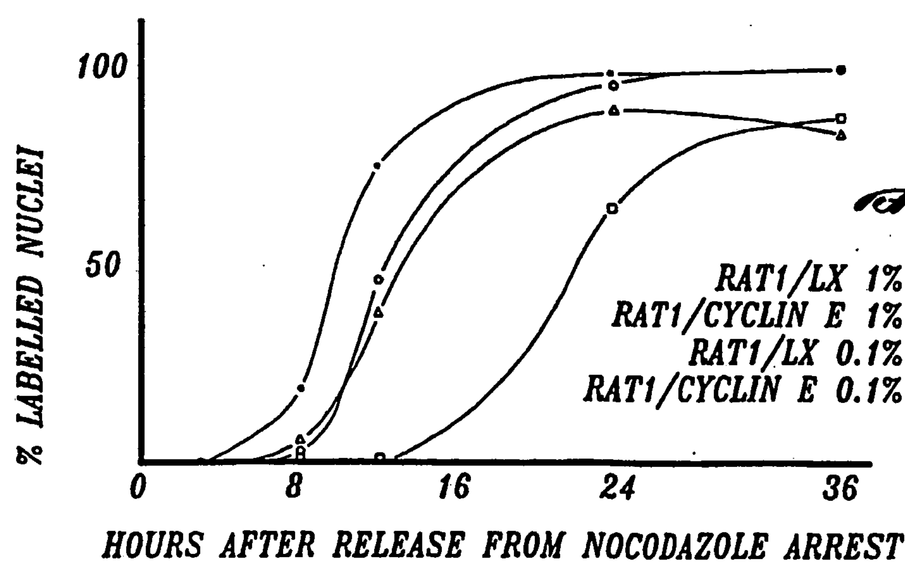


Fig. 18B.

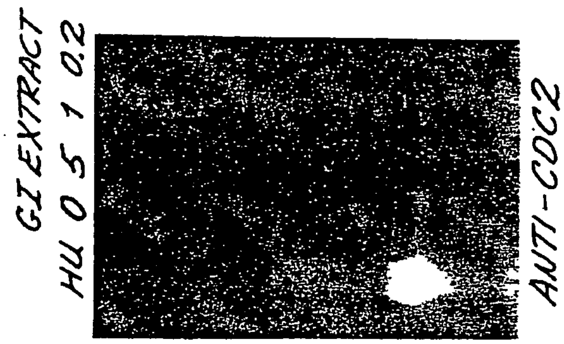


Fig.16C.

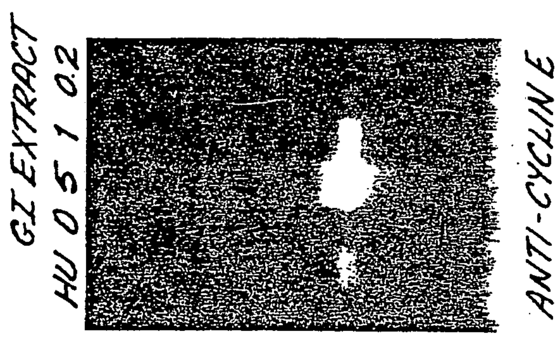


Fig.16B.

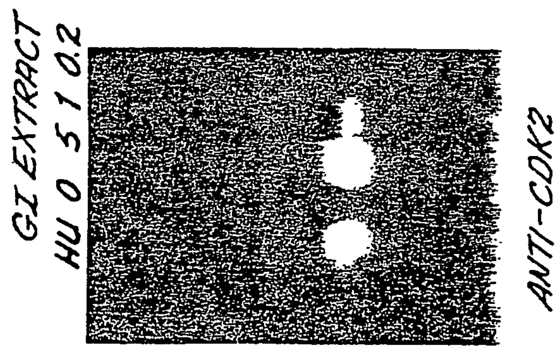


Fig.16A.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07866

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 21/04; C12N 15/70, 5/06; C12P 21/00, 19/34; C07K 13/00
US CL :536/27; 435/320.1, 240.4, 6, 91; 530/350, 387.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 435/320.1, 242., 6, 91; 530/350, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: cyclin E, dna, antisence, cell division

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E Y	Cell, Volume 70, issued 18 September 1992, P. H. Hinds et al., "Regulation of retinoblastoma Protein Functions by Ectopic Expression of Human Cyclins", pages 993-1006, entire document.	1-11,15-20,31 12-14,21-30
X,P Y	Cell, Volume 66, No. 6, issued 20 September 1991, A. Koff et al., "Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family", pages 1217-1228, entire document.	1-11,15-21,31 12-14,22-30
X,P Y	Cell, Volume 66, No. 6, issued 20 September 1991, D. J. Lew et al., "Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast", pages 1197-1206, entire document.	1-11,15-21,31 12-14,20-30
Y	Journal of Cell Science, Volume 99, issued 1991, J. H. A. Nugent et al., "Conserved structural motifs in cyclins identified by sequence analysis", pages 669-674, entire document.	1-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing f the international search report
16 November 1992	02 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LISA T. BENNETT
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

Date: October 11, 2000

To: Thermopylae Funding Corp.
C ntact: Andrew L. Stidd; Phone No.: (212) 302-5151; Facsimile No.: (212) 302-8767; with a copy to Canadian Imperial Bank of Commerce, New York Agency, as Administrator, Attn: John Rozario; Phone No.: (212) 856-4250; Facsimile No.: (212) 856-6526

From: CIBC World Markets plc
Contact: Gina S. Ghent
Phone No.: (212)-856-6538
Facsimile No.: (212)-856-6098

Re: Master Loan Swap Confirmation

The purpose of this letter agreement (this "Master Confirmation") is to set forth and confirm certain terms and conditions of transactions CIBC World Markets plc ("CIBC"), and you ("Counterparty") anticipate entering into (each, to be, a "Transaction," collectively, to be, the "Reference Loan Portfolio" or the "Transactions").

The definitions and provisions contained in the 1991 ISDA Definitions (as supplemented by the 1998 Supplement) (collectively, the "Definitions") as published by the International Swaps and Derivatives Association, Inc. ("ISDA") are incorporated into this Master Confirmation. In the event of any inconsistency between those definitions and provisions and this Master Confirmation, this Master Confirmation will govern. Each party represents and warrants to the other that (i) it is duly authorized to enter into a Transaction and to perform its obligations thereunder and (ii) the person executing this Master Confirmation is duly authorized to execute and deliver it.

This Master Confirmation supplements, forms part of, and is subject to, the Master Agreement dated as of October 11, 2000, as amended and supplemented from time to time (the "Master Agreement") between us. This letter agreement constitutes a "Confirmation" as referred to in the Master Agreement. All provisions contained in the Master Agreement shall govern this Master Confirmation except as expressly set forth herein.

This Master Confirmation relates to separate Transactions. The Master Confirmation may not be modified in any way, except upon mutual agreement between us, to be promptly evidenced by a written agreement in the form substantially similar to this Master Confirmation and signed by (i) an authorized representative of each party to this Master Confirmation. Each Transaction shall have the terms specified below, as supplemented by a single Reference Loan Annex (in such form substantially similar to that as Annex A) attached to a separate letter agreement to be provided in a timely manner upon Transaction execution (for each Transaction, its "Reference Loan Annex")":

I. <u>Transaction Terms</u>.

Notional Amount:	On any date, the Aggregate Commitment as in effect on such date.
Trade Date:	As specified in the Reference Loan Annex.
Effective Date:	As specified in the Reference Loan Annex.
Termination Date:	The earliest of (a) the earlier of (i) the maturity date of the latest maturing Revolving Credit Note or (ii) the date on which the Revolving Credit Notes are redeemed or otherwise paid in full;

(b) the Program Termination Date; (c) the date on which the Notional Amount has been reduced to zero; (d) the Scheduled Termination Date; (e) the Credit Event Notification Date, if any; and (f) any Early Optional Termination Date.

Program Termination Date:	The earliest of (a) the day that is three (3) years following the date of this Master Confirmation and (b) the day on which all Transactions governed by this Master Loan Confirmation have been terminated.
Period End Dates:	For the purposes of Loan Receiver Payments, (a) (i) the Initial Maturity Date of each Revolving Credit Note, and (ii) thereafter, the three-month anniversary date of each such preceding Period End Date and (b) the Termination Date (each, a "Loan Receiver Period End Date"). For the purposes of Loan Payer Payments, (a) the last day of each month during the term of a Transaction; <u>provided</u>, <u>however</u>, if no cash payment was made on a Reference Loan since such immediately preceding Period End Date (or Effective Date, if applicable) then such day shall not be a Period End Date for such Transaction and (b) the Termination Date (each, a "Loan Payer Period End Date").
Calculation Period:	For the purposes of Loan Payer Payments, each period from, and including, one Loan Payer Period End Date to, but excluding, the next following applicable Loan Payer Period End Date during the term of a Transaction, except that (a) such initial Calculation Period will commence on, and include the Effective Date, and (b) such final Calculation Period will end on, but exclude, the Termination Date (each, a "Loan Payer Calculation Period"). For the purposes of Loan Receiver Payments, each period from, and including, one Loan Receiver Period End Date to, but excluding, the next following applicable Loan Receiver Period End Date during the term of a Transaction, except that (a) such initial Calculation Period will commence on, and include the Effective Date, and (b) such final Calculation Period will end on, but exclude, the Termination Date (each, a "Loan Receiver Calculation Period").
Payment Dates:	(a) For the purposes of Loan Payer Payments, the second Business Day after each Loan Payer Period End Date (each, a "Loan Payer Payment Date") and (b) for the purposes of Loan Receiver Payments, the Loan Receiver Period End Date (each, a "Loan Receiver Payment Date").
Business Day:	The following cities are specified for use in the definition of "Business Day":

New York

Business Day Convention: Following

Loan Payer: Thermopylae Funding Corp.

Loan Receiver: CIBC

Loan Payer Payments:

(a) On each Loan Payer Payment Date the Loan Payer will pay to the Loan Receiver the sum of the following, in each case calculated by the Calculation Agent as of the immediately preceding Loan Payer Period End Date for the Loan Payer Calculation Period then ending:

(i) an amount equal to the Paid Interest and Fee Amount with respect to such Loan Payer Calculation Period; and

(ii) an amount equal to the Reduction Amount for such Loan Payer Calculation Period, if such Reduction Amount is a positive number; and

(iii) an amount equal to the Collateral Interest Amount.

(b) In addition, on the Final Payment Date the Loan Payer will pay to the Loan Receiver the sum of the following:

(i) an amount, calculated by the Calculation Agent as of the Termination Date, equal to the Accrued Interest and Fee Amount; <u>provided</u>, <u>however</u>, that the Loan Payer shall have no obligation under this clause (b)(i) if a Reference Loan Default or Credit Event exists as of the Termination Date or the Final Payment Date;

(ii) an amount equal to the Maturity Payment, if the Maturity Payment is a positive number; and

(iii) an amount equal to the Collateral Interest Amount.

Loan Receiver Payments:

(a) On each Loan Receiver Payment Date the Loan Receiver will pay to the Loan Payer the sum of the following, in each case calculated by the Calculation Agent as of the immediately preceding Loan Receiver Period End Date for the Loan Receiver Calculation Period then ending:

(i) an amount equal to the sum of the accrued and unpaid interest in respect of a principal amount of the Revolving Credit Notes equal to the

Reference Loan Funded Amount for such Loan Receiver Calculation Period;

(ii) an amount that, together with the amounts payable under all other Transactions to which this Master Confirmation relates, is equal to all accrued and unpaid commitment fees due and payable under the Revolving Credit Notes;

(iii) an amount equal to the absolute value of the Reduction Amount with respect to such Loan Receiver Calculation Period, if such Reduction Amount is a negative number, provided, however, that the amounts due by the Loan Receiver hereunder shall be limited to amount of the Collateral posted pursuant to the terms hereof; and

(iv) an amount that, together with the amounts payable under all other Transactions to which this Master Confirmation relates, is equal to the Administrative Expenses for such Loan Receiver Calculation Period.

(b) In addition, on the Final Payment Date the Loan Receiver will pay to the Loan Payer the sum of the following:

(i) an amount calculated by the Calculation Agent as of the Termination Date that, together with all other Transactions to which this Master Confirmation relates and which are terminated on such Final Payment Date, is equal to the sum of the accrued and unpaid interest in respect of any portion of the Revolving Credit Notes being repaid on such date for such Loan Receiver Calculation Period;

(ii) an amount that, together with the amounts payable under all other Transactions to which this Master Confirmation relates, is equal to all accrued and unpaid commitment fees due and payable under the Revolving Credit Notes;

(iii) an amount equal to the absolute value of the Maturity Payment, if the Maturity Payment is a negative number, provided, however that the amounts due by the Loan Receiver hereunder shall be limited to amount of the Collateral posted pursuant to the terms hereof; and

(iv) an amount that, together with the amounts payable under all other Transactions to which this Master Confirmation relates, is equal to the Administrative Expenses for such Loan Receiver Calculation Period.

Recovered Amounts:

(a) In the event that any payment made with respect to the Reference Loan is required to be repaid or returned to the Reference Party or any other person (including, without limitation, any bankruptcy trustee for the Reference Party), then (1) each payment obligation under this Transaction that preceded such repayment or return shall be recomputed by the Calculation Agent as if such repaid or returned amount had not been paid and (2) any additional amount required to be paid by the Loan Payer or the Loan Receiver solely as a result of such recomputation shall be paid to the other party hereto within two Business Days after such other party's demand therefor.

(b) In the event (i) the Loan Payer is not required to make a payment of Accrued Interest and Fee Amount pursuant to clause (b)(i) under the section "Loan Payer Payments" due to the existence of a Reference Loan Default, and (ii) a payment in respect of such Accrued Interest and Fee Amount is subsequently made on the Reference Loan, and (iii) after such payment is made, the Loan Receiver so notifies the Loan Payer in writing, then the Loan Payer shall remit such amount to the Loan Receiver. Notwithstanding the aforementioned notice requirement, if a payment is subsequently made on the Reference Loan, and the Loan Payer has been able, in a commercially reasonable manner, to identify such payment, then the Loan Payer shall remit such amount to the Loan Receiver.

(c) The obligations of the parties under this "Recovered Amounts" section shall survive any termination of this Transaction.

Calculation Agent:

The Calculation Agent with respect to this Transaction shall be CIBC, provided however, that in the event that the Loan Receiver defaults in its obligations to pay Loan Receiver Payments hereunder, the Calculation Agent shall be Coöperatieve Centrale Raiffeisen-Boerleenbank B.A. The Calculation Agent will determine all amounts on the basis of information from sources recognized as official sources of such information as it pertains to the Reference Loans. Nothing herein shall be construed to require that the Calculation Agent have any interest in the Reference Loan or to require the Calculation Agent to verify any

information received by it.

Sale of Reference Loans: Unless a Reference Loan has been previously repaid or has otherwise matured, on the Termination Date the Loan Payer shall cause the applicable Reference Loan to be sold.

II. Definitions.

"Accrued Commitment Fee Amount" means, as of the Termination Date, the total amount of commitment or facility availability fees that accrued in respect of the Reference Loan pursuant to the terms of the Credit Agreement during the term of the Transaction, but only to the extent that such fees remain unpaid as of such date.

"Accrued Interest Amounts" means, as of the Termination Date, the total amount of interest that accrued in respect of the Reference Loan pursuant to the terms of the Credit Agreement during the term of the Transaction, but only to the extent that such interest remains unpaid as of such date.

"Accrued Interest and Fee Amount" means, as of the Termination Date, the sum of (without duplication) any Accrued Interest Amount, Accrued Commitment Fee Amount and Accrued Other Fee Amount (excluding any amounts accrued as yield protection or to compensate the Reference Lenders for changes in capital or reserve requirements or similar events that decrease the profitability to the Reference Lenders of making or committing the make the Reference Loan).

"Accrued Other Fee Amount" means, as of the Termination Date, the total amount of any fees (other than any Accrued Commitment Fee Amount, any Accrued Interest Amount or any accrued Administrative Fees) that accrued pursuant to the Credit Agreement during the term of the Transaction, but only to the extent that such fees remain unpaid as of such date.

"Administrative Expenses" has the meaning set forth in the Security Agreement.

"Administrative Fees" means fees paid to the lender or agent under any Credit Agreement as compensation for acting as agent under or for providing administrative services with respect to the Credit Agreement or the Reference Loan if a change to such fee would not, under the terms of the Credit Agreement, require the vote of all of the lenders under the Credit Agreement.

"Advance" has the meaning set forth in each Revolving Note Purchase Agreement.

"Aggregate Commitment" means, as of any date, the aggregate outstanding commitment (whether funded or unfunded) to extend credit to the Reference Party under the Credit Agreement attributable to the Reference Loan.

"Bankruptcy Event" means that the party (i) is dissolved (other than pursuant to a consolidation amalgamation or merger); (ii) becomes insolvent or is unable to pay its debts or fails or admits in writing its inability generally to pay its debts as they become due; (iii) makes a general assignment, arrangement or composition with or for the benefit of its creditors; (iv) institutes or has instituted against it a proceeding seeking judgment of insolvency or bankruptcy or any other relief under any bankruptcy or insolvency law or other similar law affecting creditors' rights, or a petition is presented for its winding-up or liquidation, and, in the case of any such proceeding or petition instituted or presented against it, such proceeding or petition (x) results in a judgment of insolvency or bankruptcy or the entry of an order for relief or the making or an order for its winding-up or liquidation or (y) is not dismissed, discharged stayed

or restrained in each case within 30 days of the institution or presentation thereof, (v) has a resolution passed for its winding-up, official management or liquidation (other than pursuant to a consolidation, amalgamation or merger); (vi) seeks or becomes subject to the appointment of an administrator, provisional liquidator, conservator, receiver, trustee, custodian or other similar official for it or for all or substantially all its assets; (vii) has a secured party take possession of all or substantially all its assets or has a distress, execution, attachment, sequestration or other legal process levied, enforced or sued on or against all or substantially all its assets and such secured party maintains possession, or any such process is not dismissed, discharged, stayed or restrained, in each case within 30 days thereafter; (viii) causes or is subject to any event with respect to it which, under the applicable laws of any jurisdiction, has an analogous effect to any of the events specified in clauses (i) to (vii) (inclusive); or (ix) takes any action in furtherance of, or indicating its consent to, approval or, or acquiescence in, any of the foregoing acts.

"Cash" means the lawful currency of the United States of America.

"Collateral" means all collateral pledged pursuant to Article V hereof. The Collateral may be commingled with any other assets of the Counterparty in its sole discretion without notice to CIBC and may be used by the Counterparty (including, without limitation, by way of sale, pledge, repledge, rehypothecation or assignment) for its own account and at its own risk subject to the obligation of the Counterparty to return such Collateral CIBC upon fulfillment of all of the obligations of CIBC under this Master Confirmation.

"Collateral Interest Amount" means the interest earned on the Collateral as calculated by the Calculation Agent for the relevant Loan Payer Calculation Period, such interest calculated using an interest rate (i) mutually agreed upon by CIBC and Counterparty and (ii) greater than or equal to the lowest published USD-Federal Funds-H.15 Rate Option during such Loan Payer Calculation Period.

"Credit Agreement" means the documentation relating to the Reference Loan, as amended from time to time.

"Credit Event" means the occurrence of any of the following events:

(a) Publicly Available Information exists confirming the existence or occurrence of a Bankruptcy Event of the Reference Party;

(b) Publicly Available Information exists confirming the existence or occurrence of a Restructuring and the effect of such Restructuring is that the terms of the restructured Obligation are, overall, materially less favorable from a credit or risk perspective to the holder of such Obligation; or

(c) Publicly Available Information exists confirming the existence or occurrence of a Payment Default.

"Credit Event Notification Date" means the Business Day which occurs the earlier of (a) thirty (30) days after the Business Day on which the Loan Payer notifies the Loan Receiver orally (confirmed in writing) or in writing that a Credit Event has occurred and that the Loan Payer is exercising its option to terminate the Transaction and (b) any Business Day following such notice of a Credit Event on which Loan Receiver notifies the Loan Payer of the Termination Date.

"Early Optional Termination Date" means the date that the Loan Receiver elects to terminate the Transaction pursuant to Part III of this Confirmation.

"Final Payment Date" means (a) (i) for Loan Payer Payments, if there are other outstanding Transactions governed by this Master Confirmation, the second Business Day following what would have been the next Loan Payer Period End Date if the Transaction had not been terminated and (ii) for Loan Receiver Payments, if there are other outstanding Transactions governed by this Master Confirmation, the Business Day that would have been the next Loan Receiver Period End Date or (b) if there are no other outstanding Transactions governed by this Master Confirmation, the second Business Day following the Termination Date.

"Fiscal Agency Agreement" means the Fiscal Agency Agreement between the Counterparty, as Issuer and CIBC, as Fiscal Agent, as amended, supplemented or modified from time to time.

"Initial Price" means the percentage designated as such in the Reference Loan Annex (which excludes accrued but unpaid interest as of the Effective Date).

"Maturity Payment" means the Notional Amount on the Termination Date multiplied by the excess, if any, of the Maturity Price over the Initial Price (in which case the Maturity Payment will be expressed as a positive number), or by the excess, if any, of the Initial Price over the Maturity Price (in which case the Maturity Payment will be expressed as a negative number), as the case may be.

"Maturity Price" means the Sale Price, or if the Reference Loan is not sold, zero.

"Net Swap Payment" means, on any Payment Date, with respect to any Transaction or group of Transactions, an amount equal to the aggregate of all Loan Payer Payments payable on such Payment Date minus the aggregate of all Loan Receiver Payments payable on such Payment Date, in each case in respect of such Transactions.

"Obligations" means the Reference Loan.

"Paid Commitment Fee Amount" means, with respect to any period, the total amount of commitment or facility availability fees that have been paid by or on behalf of the Reference Party under the Credit Agreement during such period, but only to the extent such fees have accrued pursuant to the Credit Agreement during the term of the Transaction and prior to the end of such period.

"Paid Interest Amount" means, with respect to any period, the total amount of interest that has been paid by or on behalf of the Reference Party during such period, but only to the extent that such interest has accrued in respect of the Reference Loan pursuant to the terms of the Credit Agreement during the term of the Transaction and prior to the end of such period.

"Paid Interest and Fee Amount" means, with respect to any period, the sum of (without duplication) any Paid Interest Amount, Paid Commitment Fee Amount and Paid Other Fee Amount with respect to such period (excluding any amounts paid by or on behalf of the Reference Party as yield protection or to compensate the Reference Lenders for changes in capital or reserve requirements or similar events that decrease the profitability to the Reference Lenders of making or committing to make the Reference Loan).

"Paid Other Fee Amount" means, with respect to any period, the total amount of any fees (other than any Paid Commitment Fee Amount or Administrative Fees) that have been paid by or on behalf of the Reference Party during such period, but only to the extent that such fees have accrued pursuant to the Credit Agreement during the term of the Transaction and prior to the end of such period.

"Payment Default" means the failure of the Reference Party (i) make any payment due under any Obligation on the due date for such payment and (ii) cure such failure prior to the expiration of all applicable grace periods.

"Portfolio Notional Amount" means the aggregate Notional Amount of all Transactions governed by this Master Confirmation.

"Publicity Available Information" means information which has been published in or on any two or more of the New York Times, The Wall Street Journal, The Financial Times, The London Gazette, any Reuters Screen, any Telerate Screen or any other internationally recognized published or electronically displayed financial news source regardless of whether the reader or user thereof pays a fee to obtain such information; *provided, however*, that if any party hereto or any of its respective affiliates is cited as the sole source for such information, then such information shall not be deemed to be Publicly Available Information.

"Reduction Amount" means, with respect to any period, (i) the aggregate amount of any reduction in the Aggregate Commitment during such period multiplied by (ii) the excess, if any, of 100% over the Initial Price (in which case the Reduction Amount will be expressed as a positive number), or of the Initial Price over 100% (in which case the Reduction Amount will be expressed as a negative number), as the case may be.

"Reference Lenders" means the lenders of the Reference Loan pursuant to the Credit Agreement.

"Reference Loan" means the loan transaction described on the Reference Loan Annex with an Aggregate Commitment on the Trade Date equal to the Initial Notional Amount specified in the Reference Loan Annex. Each Reference Loan shall meet the criteria set forth on Schedule A hereto on the applicable Trade Date.

"Reference Loan Accrued Net Swap Payments" means all accrued but unpaid Loan Payer Payments minus all accrued but unpaid Loan Receiver Payments relating to a Transaction governed by this Master Confirmation, as calculated by the Calculation Agent.

"Reference Loan Default" means (i) any "event of default" or similar event with respect to the Reference Loan, (ii) any event which with the giving of notice, lapse of time, or both, would constitute such an "event of default" or similar event, or (iii) any other default (other than a default by a Reference Lender under the Credit Agreement) with respect to the Reference Loan.

"Reference Loan Funded Amount" is an amount equal to the outstanding Advance for the applicable Reference Loan multiplied by the Initial Price, each as specified in the Reference Loan Annex.

"Reference Loan Market Value" means the current market value of a Reference Loan as determined by the Calculation Agent based on information provided by market sources that will include, but not be limited to, Loan Pricing Corporation.

"Reference Party" means each entity specified as such in the Reference Loan Annex.

"Reference Portfolio Accrued Net Swap Payments" means the aggregate amount of all Reference Loan Accrued Net Swap Payments, as calculated by the Calculation Agent.

"Reference Portfolio Funded Amount" means the aggregate amount of the Reference Loan Funded Amounts.

"Reference Portfolio Market Value" means the aggregate amount of all Reference Loan Market Value, as calculated by the Calculation Agent.

"Reference Portfolio Notional Amount" means the aggregate notional amount of the Reference Loan Portfolio.

"Restructuring" means that the Reference Party agrees with its creditors to a waiver, deferral, restructuring, rescheduling, standstill, exchange or other adjustment with respect to any Obligation.

"Revolving Note Purchase Agreement" means, each Revolving Note Purchase Agreement among the Counterparty, as Issuer, CIBC, as Revolving Note Agent, and the purchaser named therein.

"Revolving Credit Note" means each Note issued by the Loan Payer pursuant to the terms of the Fiscal Agency Agreement and each Revolving Note Purchase Agreement, each of which Revolving Credit Notes are identified in the Reference Loan Annex.

"Sale Price" means the fair market value of a participation or assignment in the Reference Loan (exclusive of any Accrued Interest and Fee Amounts), expressed as a percentage of the Aggregate Commitment, as of 10:00 A.M. (New York time) on or about the tenth Business Day prior to the Termination Date (the "Valuation Date"), as determined in a commercially reasonable manner by the Calculation Agent, provided, however, that if the Reference Loan is not trading on standard LSTA documentation then the Valuation Date shall be on or about the twenty-fifth Business Day prior to the Termination Date. The Calculation Agent shall immediately notify each party hereto of its determination of the Sale Price either by facsimile (confirmed telephonically) or in writing. In the event that any party hereto disputes the Calculation Agent's determination, such party shall immediately notify the Calculation Agent thereof either by telephone (confirmed in writing) or in writing, and the Sale Price shall equal the highest bid price for a participation or assignment in the Reference Loan (based on documentation acceptable to the Calculation Agent and exclusive of Accrued Interest and Fee Amounts) in an amount equal to the Notional Amount (expressed as a percentage of the Notional Amount) after soliciting bids from (i) at least three nationally recognized brokers and, (ii) at the Loan Receiver's option, the Loan Receiver. In the event that no bids are received, the Calculation Agent shall determine the Sale Price in a commercially reasonable manner.

"Scheduled Termination Date" has the meaning specified in the Reference Loan Annex.

III. Early Optional Termination.

On any Business Day on or after the Effective Date, the Loan Receiver may elect to terminate an outstanding Transaction covered by this Master Confirmation by delivering irrevocable notice to the Loan Payer in writing or telephonically (promptly confirmed in writing) not less than (a) ten (10) Business Days prior to the relevant Early Optional Termination Date specified in such notice provided, however, that if the Reference Loan is not trading on standard LSTA documentation, notice shall be not less than twenty-five (25) Business Days. The obligations of the parties under this section shall survive any such termination(s).

IV. Representations.

(a) Representations of the Counterparty. The representations and warranties of the Counterparty set forth in the Agreement (including, without limitation, in Part 5 of the Schedule to the Agreement) shall be deemed to be repeated as of the Trade Date. In addition, BY ENTERING INTO THIS SWAP TRANSACTION, THE COUNTERPARTY IS DEEMED TO HAVE REPRESENTED

THAT ALL OF THE FOLLOWING STATEMENTS WITH RESPECT TO IT ARE TRUE AND CORRECT ON THE TRADE DATE, AND THAT IT ACKNOWLEDGES THAT CIBC HAS RELIED ON SUCH REPRESENTATIONS AND WARRANTIES IN ENTERING INTO THIS TRANSACTION.

(i) The Counterparty has sufficient knowledge, experience and professional advice to make its own legal, tax, accounting and financial evaluation of the merits and risks of entering into this Transaction and in doing so is not relying upon the views or advice of, or any information (including information with respect to the Reference Party) provided by CIBC or any affiliate of CIBC. Further, the Counterparty recognizes that this Transaction is a high yield loan swap. If a Credit Event or Reference Loan Default occurs, the payments made by the Loan Receiver in this Transaction, if any, may be significantly greater than the total of those payments received by the Loan Payer and may exceed the value of any collateral pledged by the Loan Receiver.

(ii) The Counterparty has itself been, and will at all times continue to be, solely responsible for making its own independent appraisal of and investigation into the financial condition, prospects, creditworthiness, affairs, status and business of the Reference Party.

(iii) To the best of its knowledge, the Counterparty has not received actual notice of the existence of any Reference Loan Default from the Reference Party or any Reference Lender.

(b) Representations of CIBC. The representations and warranties of CIBC set forth in the Agreement (including, without limitation, in Part 5 of the Schedule to the Agreement) shall be deemed to be repeated as of the Trade Date. In addition, CIBC represents as of the Trade Date that CIBC is an "eligible swap participant" within the meaning of 17 C.F.R. § 35.1(b)(2).

V. Collateral Provisions.

Upfront Collateral: CIBC hereby pledges to Counterparty and grants to Counterparty a security interest in and a lien upon the Collateral, in the Cash amount set forth on the Reference Loan Annex, which amount shall be an amount equal to not less than 15% of the Reference Loan Funded Amount, together with any investments in which such amount may be invested from time to time, all distributions, revenues, substitutions, replacements, benefits, profits and proceeds (including proceeds as defined in the Uniform Commercial Code as in effect in the State of New York), in whatever form, of any of the foregoing (together, the "Collateral"). Such Collateral shall be delivered to the account designated in writing by the Counterparty.

Return of Collateral: (i) If, at any time, the Collateral exceeds 15% of the Reference Portfolio Funded Amount (as determined by the Calculation Agent) at the request of CIBC, the Counterparty shall return to CIBC an amount equal to such excess or such lesser amount

as requested by CIBC.

(ii) On two (2) Business Days request from CIBC following the Final Payment Date of any Transaction, the Counterparty shall return to CIBC the Collateral, or such lesser amount as requested by CIBC.

VI. Account Details.

Payments to CIBC:

Account for Payments: Chase Manhattan Bank NY
ABA # 021 000 021
Account # 544-708-234
Account Name: CIBC London plc

Payments to Counterparty:

Account for Payments: Bank of New York
ABA # 021 000 018
Acct # 890-0331-046
Acct name: CIBC New York Agency
For Further Credit to: Collection Account – CIBC, as Collateral Agent for the Secured Parties, Account: 9002504715

VII. Other Provisions.

Settlement Amount: Notwithstanding the provisions of Section 14 of the Master Agreement, if CIBC designates an Early Termination Date under Section 6(a) or (b) of the Master Agreement, and the Counterparty is the Defaulting Party or the Affected Party, the Early Termination Date shall be deemed to be the Termination Date and the Transaction shall settle in accordance with the Master Confirmation governing the Final Payment Date.

Transfer: Unless otherwise specified in the Master Agreement, neither the Transaction nor any interest or obligation in or under the Transaction may be transferred (whether by way of security or otherwise) by either party without the prior written consent of the other party. Any purported transfer that is not in compliance with this provision will be void.

Governing Law: The Laws of the State of New York (without reference to the choice of law doctrine).

Confirmation Counterparts: This Confirmation may be executed in one or more counterparts, either in original or facsimile form,

each of which shall constitute an original and all of which together shall constitute one and the same agreement. When executed by the parties through facsimile transmission, this Confirmation shall constitute the original agreement between the parties and the parties hereby adopt the signatures printed by the receiving facsimile machine as the original signatures of the parties.

Broker/Arranger:	None

VIII. Offices.

(a) The Office of CIBC for the Transaction is Cottens Centre, Cottons Lane, 2nd Fl., London SE1 2QA.

(b) The Office of Counterparty for the Transaction is c/o Global Securitization Services, LLC, 114 West 47th Street, Suite 1715, New York, New York 10017, Attention: Andrew L. Stidd, with a copy to the Administrator, CIBC, 425 Lexington Avenue, New York, New York, 10017.

Entering into a derivative transaction involves certain risks. An identification of the principal risks is provided in the CIBC World Markets Risk Disclosure Statement, which has been delivered to you. If you have not received a copy, please let us know and one will be provided to you. You should always consider those risks in determining whether to enter into derivatives transactions.

Except as if expressly agreed to by you or us in writing, neither of us has acted as advisor to the other with respect to the desirability or appropriateness of entering into the Transaction confirmed hereby or with respect to the other party's risk management needs generally. This pertains not only to the financial and market risk management risks and consequences of the confirmed or any proposed Transaction, but also to any legal, regulatory, tax, accounting and credit issues generated by such transactions, which each party must evaluate for itself and in reliance on its own professional advisors.

Please confirm that the foregoing correctly sets forth the terms of our agreement by executing the copy of this Confirmation enclosed for that purpose and returning it to us or by sending to us a letter or telex substantially similar to this letter, which letter or telex sets forth the material terms of the Transaction to which this Confirmation relates and indicates your agreement to those terms.

Yours Sincerely,

CIBC WORLD MARKETS plc

By: ________________________________

Name: Gina S. Ghent
Title: Executive Director

Confirmed as of the date first above written:

THERMOPYLAE FUNDING CORP.

By: ________________________________

Name:
Title:

REFERENCE LOAN ANNEX

GENERAL INFORMATION:

Reference Number:	
Trade Date:	
Transaction Effective Date:	[The later of (a) the tenth Business Day after the Trade Date and (b) the Business Day on which CIBC notifies the Counterparty that the Transaction is effective.]
Scheduled Termination Date:	
Collateral:	USD [________________]
Revolving Credit Notes:	[Describe Note amount, applicable Advance amount and Initial Maturity Date]

REFERENCE LOAN INFORMATION:

Initial Notional Amount:	(Aggregate Commitment Amount at Trade Date)
Reference Party:	
Reference Loan:	
Initial Price:	(excluding accrued, but unpaid interest and fees on the Effective Date, if any)

Thermopylae Reference Loan Requirements

Reference Loans: All senior secured, syndicated bank loans with demonstrated liquidity. All Reference Loans will satisfy the following criteria:

- all Reference Loans must be rated by either Moody's Investor Service, Standard & Poor's Rating Services, a division of the McGraw-Hill Companies, Inc. or Fitch
- all Reference Loans will be rated at least "B" by Fitch
- all Reference Loans will have a maturity of 10 years or less
- all Reference Loans are US$-denominated
- all Reference Loans will have only US borrowers
- all Reference Loans will accrue interest at floating rates
- no Reference Loan will have an Initial Price less than 95.00%
- no Reference Loan will comprise more than 10% of its related credit facility
- all Reference Loans must be priced by Loan Pricing Corporation

SCHEDULE
to the
Master Agreement
dated as of October 11, 2000

between CIBC World Markets plc ("Party A") and Thermopylae Funding Corp. ("Party B")

Capitalized terms used and not defined herein have the meanings specified in the Glossary of Terms attached hereto.

Part 1. Termination Provisions

(a) **"Specified Entity"** means in relation to Party A for the purpose of: -

Section 5(a)(v) (Default under Specified Transaction), Not Applicable
Section 5(a)(vi) (Cross Default), Not Applicable
Section 5(a)(vii) (Bankruptcy), Not Applicable
Section 5(b)(iv) (Credit Event Upon Merger), Not Applicable

and in relation to Party B for the purpose of: -

Section 5(a)(v) (Default under Specified Transaction), Not Applicable
Section 5(a)(vi) (Cross Default), Not Applicable
Section 5(a)(vii) (Bankruptcy), Not Applicable
Section 5(b)(iv) (Credit Event Upon Merger), Not Applicable

(b) **"Specified Transaction"** will have the meaning specified in Section 14 of this Agreement.

(c) The following Events of Default under Section 5(a) of this Agreement will not apply to Party A or Party B:

(i) The "**Breach of Agreement**" provisions of Section 5(a)(ii);

(ii) The "**Credit Support Default**" provisions of section 5(a)(iii);

(iii) The "**Misrepresentation**" provisions of Section 5(a)(iv);

(iv) The "**Default under Specified Transaction**" provisions of Section 5(a)(v); and

(v) The "**Cross Default**" provisions of Section 5(a)(vi).

(d) The **"Credit Event Upon Merger"** provisions of Section 5(b)(iv) will not apply to Party A or to Party B.

(e) The **"Automatic Early Termination"** provision of Section 6(a) will not apply to Party A or to Party B.

(f) **Payments on Early Termination.** Section 6(e) of this Agreement is amended in its entirety to provide that if an Early Termination Date is designated, irrespective of whether such Early Termination Date is designated as a result of an Event of Default or a Termination Event hereunder,

In respect of such Early Termination Date, and on the date on which a payment is due in respect thereof as set forth in Section 6(d)(ii) hereof, each party shall pay to the other party, for each Transaction then being terminated, an amount equal to that amount (as set forth in the Confirmation for such Transaction) that would have been payable by such party if such date were the Final Payment Date for such Transaction.

(g) **"Termination Currency"** means United States Dollars.

(h) **Additional Termination Event,** will not apply.

(i) **Amendment of Section 2(a)(iii).** The provisions of Section 2(a)(iii) of this Agreement do not apply with respect to payments due from Party B to Party A hereunder.

(k) **Subordination of Certain Payments due Party A.** Any amounts which would be due Party A pursuant to Section 2(e) or Section 11 of this Agreement shall be subordinated until all amounts due the Noteholders have been paid in full.

Part 2. Tax Representations

(a) **Payer Representation.** For the purpose of Section 3(e) of this Agreement, Party A and Party B will each make the following representation: -

It is not required by any applicable law, as modified by the practice of any relevant governmental revenue authority, of any Relevant Jurisdiction to make any deduction or withholding for or on account of any Tax from any payment (other than interest under Section 2(e), 6(d)(ii) or 6(e) of this Agreement) to be made by it to the other party under this Agreement. In making this representation, it may rely on (i) the accuracy of any representation made by the other party pursuant to Section 3(f) of this Agreement, (ii) the satisfaction of the agreement of the other party contained in Section 4(a)(i) or 4(a)(iii) of this Agreement and the accuracy and effectiveness of any document provided by the other party pursuant to Section 4(a)(i) or 4(a)(iii) of this Agreement and (iii) the satisfaction of the agreement of the other party contained in Section 4(d) of this Agreement, provided that it shall not be a breach of this representation where reliance is placed on clause (ii) and the other party does not deliver a form or document under Section 4(a)(iii) of this Agreement by reason of material prejudice to its legal or commercial position.

(b) **Payee Representations.** Party A and Party B make no Payee Tax Representations.

Part 3. Agreement to Deliver Documents.

For the purpose of Section 4(a)(i) and 4(a)(ii) of this Agreement, each party agrees to deliver the following documents as applicable:

(a) Tax forms, documents or certificates to be delivered are: None.

(b) Other documents to be delivered are:

PARTY REQUIRED TO DELIVER DOCUMENT	FORM/ DOCUMENT/ CERTIFICATE	DATE BY WHICH TO BE DELIVERED	COVERED BY SECTION 3(d) REPRESENTATION

Party A & B	Certificate of Incumbency and Signing Authority of each person executing any document on its behalf in connection with this Agreement	Upon execution of this Agreement and, if requested any Confirmation	Yes

Part 4. Miscellaneous

(a) **Addresses for Notices**: For the purpose of Section 12(a) of this Agreement: -

Address for notices or communications to Party A:

Address: CIBC World Markets plc
Cottons Centre, 2nd Floor
Cottons Lane
London SE1 2QA

with a copy to:

Canadian Imperial Bank of Commerce, New York Agency
425 Lexington Avenue
New York, New York 10017
Attention: Gina S. Ghent
Facsimile No.: 212-856-6098
Telephone No.: 212-856-6538

Address for notices or communications to Party B:

Address: Thermopylae Funding Corporation
c/o Global Securitization Services, LLC
114 W. 47th Street, Suite 1715
New York, New York 10036
Attn: Andrew L. Stidd
Facsimile No.: 212-302-8767
Telephone No.: 212-302-5151

with a copy to:

Canadian Imperial Bank of Commerce, New York Agency
425 Lexington Avenue
New York, New York 10017
Attn: John Rozario
Facsimile No.: 212-856-6256
Telephone No.: 212-856-4250

(b) **Process Agent.** For the purpose of Section 13(c) of this Agreement:

Party A appoints as its Process Agent: Not Applicable.

Party B appoints as its Process Agent: Not Applicable.

(c) **Offices.** The provisions of Section 10(a) will apply to this Agreement.

(d) **Multibranch Party.** For the purpose of Section 10(c) of this Agreement:-

Party A is not a Multibranch Party.

Party B is not a Multibranch Party.

(e) **Calculation Agent.** The Calculation Agent will be Party A unless (i) an Event of Default has occurred and is continuing with respect to Party A or (ii) otherwise specified in a Confirmation in relation to the relevant Transaction. Party A's failure to perform its obligations as Calculation Agent hereunder shall not be construed as an Event of Default or Termination Event.

(f) **Credit Support Document.** Not applicable to either Party A or Party B.

(g) **Credit Support Provider.** Not applicable to either Party A or Party B.

(h) **Governing Law.** This Agreement will be governed by and construed in accordance with the laws of the State of New York without reference to choice of law doctrine.

(i) **Netting of Payments.** Subparagraph (ii) of Section 2(c) of this Agreement shall not apply and netting across all Transactions will be made.

(j) **"Affiliate"** will have the meaning specified in Section 14 of this Agreement with respect to Party A and will mean "none" with respect to Party B.

Part 5. Other Provisions

(a) **Definitions.** Unless otherwise specified in a Confirmation, this Agreement and the Transaction contemplated hereby between the parties are subject to the 1991 ISDA Definitions (as supplemented by the 1998 Supplement) as published by the International Swap Dealers Association, Inc. (the "Definitions"), and will be governed in all relevant respects by the provisions set forth in the Definitions, without regard to any amendment to the Definitions subsequent to the date hereof. The provisions of the Definitions are incorporated by reference in and shall be deemed a part of this Agreement. In the event of any inconsistency between the provisions of this Agreement and the Definitions, this Agreement will prevail.

(b) **Confirmations.** Each Confirmation shall be substantially in the form of Annex A to the Agreement.

(c) **Consent to Telephonic Recording.** Each party hereto consents to the monitoring or recording, at any time and from time to time, by the other party of any and all communications between officers or employees of the parties, waives any further notice of such monitoring or recording, agrees to notify its officers and employees of any such monitoring or recording, and agrees that any such tape recordings may be submitted in evidence in any Proceedings relating to this Agreement and any Transaction hereunder.

(d) **No Petition for Bankruptcy.** Party A shall not petition for the bankruptcy or insolvency of Party B at any time prior to one year and one day after payment in full of outstanding Notes. The agreement of Party A hereunder shall survive the termination of this Agreement.

(e) **Severability.** Any provision of this Agreement which is prohibited or unenforceable in any jurisdiction shall, as to such jurisdiction, be ineffective to the extent of such prohibition or unenforceability without invalidating the remaining provisions of the Agreement or affecting the

validity or enforceability of such provision in any other jurisdiction unless such severance shall substantially impair the benefits, including netting benefits, of the remaining portions of this Agreement or changes the reciprocal obligations of the parties.

(f) **Information Relating to the Reference Loans.** Party A may request Party B to and Party B shall obtain any reports, notices, financial statements or other information in respect of any Reference Loan which the holders of such Reference Loan are entitled to receive upon request pursuant to the terms of such Reference Loan.

(g) **Further Assurances.** Promptly following a demand made by Party A, Party B will execute, deliver, file and record any financing statement, specific assignment or other document and take any other action that be necessary or desirable and reasonably requested by Party A to create, preserve, perfect or validate any security interest or lien granted hereunder to enable Party A to enforce its rights under this Agreement or to effect or document a release of such security interest.

(h) **Security Interest.** Party A acknowledges and agrees that Party B has granted a security interest in its rights against Party A hereunder to the Collateral Agent on behalf of the Secured Parties and that under certain circumstances as set forth in the Security Agreement the Collateral Agent will have the right to act on behalf of the Party B hereunder.

(i) **Limited Recourse.**

(i) If and to the extent that the Available Funds are insufficient to pay the amount otherwise payable by Party B to Party A, such unpaid obligation shall not constitute a "claim" (within the meaning of Section 101(5) of the Bankruptcy Code) against Party B and shall be payable on the next succeeding date when the Available Funds are sufficient to pay such amount.

(ii) No recourse shall be had for the payment of any amount owing in respect of the obligations appertaining hereto against any officer, director, employee, stockholder, director or incorporator of Party B or any successor or affiliate of Party B or the Administrator.

(j) **Confidential Information.** Each party may share any information concerning the other party with any of its Affiliates.

(k) **Transfer and Assignment.** Party A and Party B each agree not to transfer or assign or consent to the transfer or assignment of this Agreement except upon written agreement.

(l) **Waiver of Jury Trial.** EACH PARTY HEREBY IRREVOCABLY WAIVES ANY AND ALL RIGHT TO TRIAL BY JURY IN ANY PROCEEDINGS.

(m) **Amendments.** No amendment, modification, supplement or waiver of this Agreement will be effective unless in writing and signed by each of the parties hereto.

IN WITNESS WHEREOF, Party A and Party B have caused this Schedule to be duly executed as its act and deed as of the date first written above.

CIBC WORLD MARKET plc

By______________________________
Name:
Title:

THERMOPYLAE FUNDING CORP.

By______________________________
Name:
Title:

ANNEX A

FORM OF CONFIRMATION

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SECURITY AGREEMENT

THIS SECURITY AGREEMENT (this "Agreement") is hereby entered into as of October 11, 2000, by and between THERMOPYLAE FUNDING CORP., a corporation organized under the laws of Delaware (the "Issuer"), and CANADIAN IMPERIAL BANK OF COMMERCE, NEW YORK AGENCY ("CIBC" or the "Collateral Agent"), for the benefit of the Secured Parties (as defined below).

RECITALS

WHEREAS, the Issuer intends to (i) issue Revolving Notes (the "Notes"), from time to time on the terms and conditions set forth in one or more Revolving Note Purchase Agreements, between the Issuer and the Noteholder named therein (each, a "Noteholder") and (ii) enter into a total rate of return swap with CIBC (the "Swap Counterparty", and together with the Noteholders, the "Secured Parties");

WHEREAS, to secure its obligations to each Secured Party, in each case subject to the payment priorities set forth herein, the Issuer wishes to pledge, to the Collateral Agent, all of the Issuer's right, title and interest in and to the Collateral (as defined herein);

WHEREAS, the Issuer desires to enter into an agreement with the Collateral Agent to effect such pledge and to further set forth the respective rights of each Secured Party to the Collateral; and

WHEREAS, CIBC is willing to act as Collateral Agent hereunder.

NOW THEREFORE, in consideration of the mutual premises and covenants contained herein, and for other good and valuable consideration the receipt of which is hereby acknowledged, the parties hereto agree, for the benefit of the Secured Parties, as follows:

AGREEMENT

SECTION 1. <u>DEFINITIONS</u>. Capitalized terms used herein (or in any certificate or other document made or delivered pursuant hereto) but not defined herein (or therein) shall have the meanings assigned to such terms in the Glossary of Terms attached hereto as Annex A. The words "hereof," "herein" and "hereunder" and words of similar import, when used in this Agreement, shall refer to this Agreement as a whole and not to any particular provision of this Agreement. Section, subsection and Exhibit references contained in this Agreement are references to Sections, subsections and Exhibits in or to this Agreement unless otherwise specified.

SECTION 2. <u>GRANT OF SECURITY INTEREST</u>.

2.1. In order to secure the full and punctual payment of, and the performance by the Issuer of all of its obligations owing from time to time to the Secured Parties, the Issuer hereby pledges, assigns, transfers and conveys to the Collateral Agent, for the benefit of the Secured Parties a continuing security interest in, and a lien upon, all of the Issuer's right, title and interest in, to and under, the following, whether now owned or hereafter acquired (collectively, the "Collateral"):

(a) all Assets at any time Purchased by the Issuer and all payments and monies that become due thereon, and any guarantees and insurance, if any, thereof, and all rights, claims, powers, privileges and remedies of the Issuer, whether arising by contract or at law or in equity or otherwise, under such Assets and all collateral and other assets or property securing the obligation of each Asset Obligor with respect to such Assets;

(b) the Master Swap Agreement (including, the schedule and all confirmations thereto), and any swap agreement which may at any time hereafter be entered into

between the Issuer and any successor Swap Counterparty (a "Successor Swap Agreement"), all rights, claims, powers, privileges and remedies of the Issuer under the Master Swap Agreement and any Successor Swap Agreement and all payments and monies that become due therein, whether arising by contract or at law or in equity or otherwise pursuant thereto;

(c) the Collateral Account, the Collection Account and the Swap Collateral Account (together, the "Accounts") and all monies, credit balances, investment property and investments from time to time held in or credited to the Accounts and all securities entitlements with respect thereto;

(d) all Asset Documentation relating to Assets Purchased by the Issuer;

(e) this Security Agreement, the Management Agreement, each Revolving Note Purchase Agreement, the Fiscal Agency Agreement and the Administration Agreement; and

(f) all distributions, revenues, substitutions, replacements, benefits, profits and proceeds (including proceeds as defined in the UCC), in whatever form, of any of the foregoing.

2.2. The rights of the Secured Parties in the Collateral are subject in all cases to the payment priorities set forth in Section 4.3 hereof.

SECTION 3. <u>DELIVERY OF COLLATERAL; ESTABLISHMENT OF SECURITIES ACCOUNTS</u>.

(a) The Issuer confirms that it has, on or prior to the date hereof, caused to be filed, in such jurisdictions as required by the Administrator, a UCC-1, satisfactory to the Administrator, naming the Collateral Agent as secured party and the Issuer as debtor.

(b) The Issuer agrees to deliver or cause to be delivered to the Collateral Agent, in accordance with the provisions hereof, each item of Collateral, now existing or hereafter arising or acquired. Each item of Collateral delivered to the Collateral Agent pursuant to any provision of this Agreement shall be delivered so that upon such delivery the Collateral Agent, on behalf of the Secured Parties, shall have a first lien on, and first priority perfected security interest in, each such item of Collateral.

(c) The Collateral Agent agrees on or prior to the Closing Date, to establish the Accounts. Each such Account shall be established in the name of the Collateral Agent, as Collateral Agent for the Secured Parties. The Collateral Agent represents and covenants for the benefit of the Secured Parties that it is (and will act with respect to each Account as) a "securities intermediary" within the meaning of the UCC and that each Account shall be a "securities account" (within the meaning of the UCC). The Collateral Agent hereby agrees that each item of property (whether investment property, financial asset, security, instrument or cash) credited to an Account shall be treated as a "financial asset" within the meaning of Section 8-102(a)(9) of the UCC.

(d) All securities or other property underlying any financial assets credited to any Account shall be registered in the name of the Collateral Agent, endorsed to the Collateral Agent or in blank or credited to another securities account or accounts maintained in the name of the Collateral Agent, and in no case will any financial asset credited to the Accounts be registered in the name of the Issuer, payable to the order of the Issuer or specially indorsed to the Issuer except to the extent the foregoing have been specially indorsed to the Collateral Agent or in blank. All property delivered to the Collateral Agent pursuant to this Agreement will be promptly credited to the applicable Account.

(e) In connection with the delivery of any item of Collateral, the Collateral Agent shall make appropriate notations on its records, and shall cause same to be made on the records of its nominees, indicating that such Collateral is held in trust pursuant to and as provided under this Agreement.

(f) The Issuer and the Collateral Agent each agree that the Issuer has no right to consent to the initiation of any "entitlement orders" (within the meaning of the UCC) by the Collateral Agent on behalf of the Secured Parties with respect to the Collateral and, except as expressly provided herein, to initiate any entitlement orders with respect to the Collateral.

(g) The Collateral Agent shall monitor the Issuer's receipt of payments from the Assets.

(h) The Issuer shall deliver to the Collateral Agent, simultaneously with the delivery of the Collateral, a schedule that identifies such Collateral in reasonable detail.

SECTION 4. COLLECTION ACCOUNT.

4.1. Establishment and Maintenance. As provided in Section 3(c), on or prior to the Closing Date, the Collateral Agent, as Securities Intermediary for the Secured Parties, shall establish a special purpose trust account which shall be a "securities account" within the meaning of the UCC (the "Collection Account") entitled "Collection Account - CIBC, as Collateral Agent for the Secured Parties." The Collection Account shall be subject to the exclusive dominion and control of the Collateral Agent, as Securities Intermediary for the Secured Parties, and the Issuer shall have no right of withdrawal from the Collection Account.

4.2. Required Deposits.

(a) The Collateral Agent shall cause the following amounts, subject to Section 11(a)(ii) hereof, to be deposited into the Collection Account:

(i) the date of each Advance, the proceeds of such Advance;

(ii) upon the Collateral Agent's receipt thereof, all payments received on any Assets held by the Collateral Agent, including any sale proceeds;

(iii) upon the Collateral Agent's receipt thereof, all payments received from the Swap Counterparty under the Master Swap Agreement; and

(iv) all other amounts received by the Issuer with respect to the Collateral.

(b) The Collateral Agent is hereby irrevocably authorized and empowered, as the Issuer's attorney-in-fact, to endorse any check or any other instrument or security presented for deposit in the Collection Account requiring the endorsement of the Issuer.

(c) If, notwithstanding the foregoing, at any time the Issuer, or any Person on behalf of the Issuer, receives any proceeds or payments required to be deposited in the Collection Account, all such amounts shall be held by the Issuer or such other Person as the agent of, and in trust for, the Collateral Agent and shall, forthwith upon receipt by the Issuer, or such other Person, be turned over to the Collateral Agent for deposit in the Collection Account, in the same form as received by the Issuer or such other Person and, if received in the form of a check, instrument or security requiring endorsement, duly endorsed on behalf of the Issuer or such other Person to the order of the Collateral Agent. If any such check, instrument or security shall not be

so endorsed, the Collateral Agent is hereby irrevocably authorized and empowered to endorse the same on behalf of the Issuer as its attorney-in-fact.

(d) The Collateral Agent has no duty or responsibility to ensure the Issuer's compliance with the provisions of this Section 4.2 and it shall have no liability if the Issuer fails to comply with such provisions.

4.3. Application of Funds in the Collection Account. On each Payment Date, subject to Section 21 hereof, the Collateral Agent shall apply funds on deposit in the Collection Account in the following order of priority:

(a) to the Fiscal Agent, on behalf of the Noteholders, an amount equal to the outstanding interest, principal and Commitment Fees, if any, due and owing on the Notes; and then

(b) to the Swap Counterparty, on behalf of the Issuer, an amount equal to the amount, if any, due the Swap Counterparty under the Master Swap Agreement; and then

(c) to pay to the respective person or persons entitled thereto, an amount equal to the aggregate Purchase Price of any Assets being Purchased on such Business Day pursuant to Section 4.4; and then

(d) to the Manager, for the payment on behalf of the Issuer of any Tax Expenses then due by the Issuer; and then

(e) pro rata, to the Collateral Agent, the Manager, the Fiscal Agent, the Administrator and the Revolving Note Agent, for any accrued and unpaid Administrative Expenses due and owing to each such Person by the Issuer; and then

(f) to the Administrator, the amount then remaining in the Collection Account, if any.

4.4. Purchase of Assets.

(a) Subject to Section 4.4(b), on any Business Day on which Assets are to be Purchased by the Issuer, the Collateral Agent will, at the written direction of the Administrator on behalf of the Issuer, withdraw available funds from the Collection Account pursuant to Section 4.3(b) and deliver such funds upon the instruction of the Administrator on behalf of the Issuer, in payment on behalf of the Issuer of the Purchase Price of such Assets.

(b) The Collateral Agent shall not release any funds from the Collection Account pursuant to Section 4.4(a) for the Purchase of Assets unless and until the Collateral Agent has received written instructions from the Administrator on behalf of the Issuer, identifying each Asset to be Purchased on such date and the principal amount and Purchase Price thereof.

(c) If any Asset Purchased by the Issuer is itself secured by any collateral, the Collateral Agent may, in its sole discretion, hold such collateral directly or through a third party custodian or collateral agent.

4.5. Sale of Assets. On the Maturity Date of any Revolving Credit Note, the Collateral Agent shall sell the related Assets in accordance with the applicable Asset Sale Procedures.

SECTION 5. COLLATERAL ACCOUNT; SWAP COLLATERAL ACCOUNT.

5.1. Collateral Account.

(a) Establishment and Maintenance. On or prior to the Closing Date, the Collateral Agent, as Securities Intermediary for the Secured Parties, shall establish a special purpose trust account which shall be a "securities account" within the meaning of the UCC (the "Collateral Account") entitled "Collateral Account – CIBC, as Collateral Agent for the Secured Parties" The Collateral Account shall be subject to the exclusive dominion and control of the Collateral Agent, as Securities Intermediary for the Secured Parties, and the Issuer shall have no right of withdrawal from the Collateral Account.

(b) Required Deposits. Each Asset acquired by the Issuer shall be acquired through and held in, or delivered to the Collateral Agent in accordance with the procedures set forth in Annex B for credit to, the Collateral Account. Each Asset delivered to the Collateral Agent pursuant to this Section shall be credited to the Collateral Account by the Collateral Agent, as Securities Intermediary for the Secured Parties, and such Asset shall be a "financial asset" within the meaning of Section 8-102(a)(9) of the UCC. The Issuer shall take any other action necessary to create in favor of the Collateral Agent a valid, perfected, first-priority security interest in each Asset under laws and regulations in effect at the time of the acquisition of such Asset, including Articles 8 and 9 of the UCC.

(c) Indorsements. In no case will an Asset be registered in the name of the Issuer, payable to the order of the Issuer or specially indorsed to the Issuer except to the extent that an Asset has been specially indorsed to the Collateral or in blank.

(d) Collections on Assets. All collections received with respect to an Asset credited to or held in the Collateral Account shall deposited to the Collection Account in accordance with Section 4 hereof.

5.2. Swap Collateral Account.

(a) Establishment and Maintenance. On or prior to the Closing Date, the Collateral Agent, as Securities Intermediary for the Noteholders, shall establish a special purpose trust account which shall be a "securities account" within the meaning of the UCC (the "Swap Collateral Account") entitled "Swap Collateral Account – CIBC, as Collateral Agent for the Noteholders." The Collateral Account shall be subject to the exclusive dominion and control of the Collateral Agent, as Securities Intermediary for the Secured Parties, and the Issuer shall have no right of withdrawal from the Swap Collateral Account.

(b) Required Deposits. Each Asset acquired by the Issuer shall be acquired through and held in, or delivered to the Swap Collateral Agent in accordance with the procedures set forth in Annex B for credit to, the Swap Collateral Account. Each Asset delivered to the Swap Collateral Agent pursuant to this Section shall be credited to the Collateral Account by the Swap Collateral Agent, as Securities Intermediary for the Secured Parties, and such Asset shall be a "financial asset" within the meaning of Section 8-102(a)(9) of the UCC. The Issuer shall take any other action necessary to create in favor of the Collateral Agent a valid, perfected, first-priority security interest in each Asset under laws and regulations in effect at the time of the acquisition of such Asset, including Articles 8 and 9 of the UCC.

(c) Indorsements. In no case will an Asset be registered in the name of the Issuer, payable to the order of the Issuer or specially indorsed to the Issuer except to the extent that an Asset has been specially indorsed to the Collateral or in blank.

(d) Collections on Assets. All collections received with respect to an Asset credited to or held in the Swap Collateral Account shall remain in the Swap Collateral Account.

SECTION 6. REPRESENTATIONS AND WARRANTIES OF THE ISSUER.

6.1. Issuer Representations and Warranties. The Issuer represents and warrants to the Collateral Agent that:

(a) The Issuer (i) is a duly organized and validly existing corporation in good standing under the laws of the State of Delaware, (ii) is duly licensed or qualified to do business and is in good standing in each jurisdiction, and is not in default under any agreement or instrument, where the failure to be so licensed or qualified, or the result of which default, respectively, could have a material adverse effect on its assets or could impair its ability to perform any of its obligations under the Program Documents and (iii) has all requisite power and authority to own or lease its properties and conduct its business as contemplated under the Program Documents and to execute, deliver and perform its obligations under the Program Documents.

(b) The execution, delivery and performance of the Program Documents will not (i) violate any existing applicable law, rule or regulation or any provision of the certificate of incorporation or by-laws of the Issuer or (ii) conflict with, result in a breach of, or constitute a default under, any terms or provisions of any indenture, mortgage or other agreement or instrument to which the Issuer is a party or by which it or any of its assets are bound.

(c) The execution, delivery and performance of the Program Documents have been duly authorized by all necessary action of the Issuer. The Program Documents have been duly executed and delivered by the Issuer and each constitutes the legal, valid and binding obligation of the Issuer, enforceable according to its terms, subject, as to enforceability, to applicable bankruptcy, insolvency, reorganization, moratorium and similar laws affecting creditors' rights generally and to general principles of equity (regardless of whether enforcement is sought in a proceeding in equity or at law).

(d) All authorizations, consents, approvals, registrations, filings, exemptions and licenses with or from governmental, regulatory authorities or any other third party which are necessary for the execution and delivery of the Program Documents to which the Issuer is a party, or for the performance by the Issuer of its obligations thereunder have been effected and obtained, and, so long as may be required for the Issuer to comply with the any Program Document, will remain in full force and effect.

(e) There are no actions or proceedings pending or threatened against the Issuer before any court or administrative agency which are likely to have a material adverse effect on the Issuer's condition or the results of its operations or its ability to perform its obligations under the Program Documents.

(f) The Collateral Agent has a valid and first priority perfected security interest in the Master Swap Agreement and any Collateral delivered to the Collateral Agent on the Closing Date for the benefit of the Secured Parties in accordance with the terms of this Agreement to the extent such security interest can be perfected by filing a financing statement or obtaining possession in accordance with the Uniform Commercial Code of the jurisdiction the law of which governs the perfection of such interest and all filing fees in connection therewith have been paid.

(g) The Issuer is not required to register as an "investment company," as defined in the Investment Issuer Act of 1940, as amended.

(h) GSS Holdings, Inc. (the "Owner") owns 100% of the voting stock of the Issuer.

(i) The Issuer has not established and does not maintain or contribute to any employee benefit plan that is covered by Title IV of the Employee Retirement Income Security Act of 1974, as amended.

(j) The principal place of business and chief executive office of the Issuer is located in New York, New York.

6.2. Additional Issuer Representations and Warranties. On and as of any date on which the Issuer Purchases any Asset (each such date, a "Purchase Date"), the Issuer shall be deemed to make the representations and warranties set forth below, as applicable, with respect to each Asset that is Purchased on such date and included in the Collateral:

(a) such asset is an Asset;

(b) to the best knowledge of the Issuer, at the time of the Sale thereof to the Issuer, the Asset was not subject to any lien or other claim, or to any material dispute, asserted offset, counterclaim or defense of any kind and if such Asset was being purchased in the secondary market, the Seller had clear and marketable title to the Asset;

(c) to the best knowledge of the Issuer, the Asset was issued in compliance with all laws and regulations applicable thereto, is valid and enforceable against the underlying obligor and is not subject to any adverse claim, proceeding or other dispute;

(d) the Issuer Purchased such Asset in a valid Sale, enforceable against the Seller of such Asset and all creditors of and purchasers from the Seller, subject only to applicable bankruptcy, insolvency, reorganization, moratorium or other similar laws affecting creditors' rights in general and to general principles of equity;

(e) the Collateral Agent has a valid and first priority perfected security interest in such Asset for the benefit of the Secured Parties in accordance with the terms of this Agreement; and

(f) none of the Asset Obligors is known by the Issuer to be insolvent or bankrupt.

SECTION 7. FURTHER ASSURANCES; COVENANTS OF THE ISSUER. The Issuer hereby covenants and agrees that, until all Notes and other obligations of the Issuer have been paid and performed in full:

(a) The Issuer will exercise due diligence in order to assure that it complies with the requirements of all applicable laws, rules, regulations and orders of any governmental authority, noncompliance with which would materially adversely affect its business, condition (financial or other), prospects, assets, property or operations.

(b) The Issuer will not enter into any business other than as is contemplated by its Certificate of Incorporation.

(c) The Issuer will not sell, transfer, exchange, pledge or otherwise dispose of any part of the Collateral except as expressly permitted by this Agreement and each Eligible Swap.

(d) The Credit Default Swap shall be in effect.

(e) The Issuer will not incur any indebtedness for borrowed money (other than in respect of the Notes) or enter into any agreements with any person pursuant to which the Issuer could incur any obligations, unless such person further agrees that:

(i) amounts payable to it by the Issuer are to be paid solely out of Available Funds;

(ii) to the extent such Available Funds are insufficient to pay amounts due such person, such unpaid obligations shall not be due and payable until the earliest date on which there Available Funds are sufficient to pay such amounts and such unpaid amounts shall not constitute a "claim" against the Issuer within the meaning of Section 101(5) of the Bankruptcy Code; and

(iii) it will not institute against, or join any other person in instituting against, the Issuer, any bankruptcy, reorganization, arrangement, insolvency or liquidation proceedings or other similar proceeding under the laws of the United States or any state of the United States, prior to the date that is one year and one day after the payment in full of all outstanding Notes.

(f) The Issuer will promptly execute and deliver and will cause to be executed and delivered all further instruments and documents, including, without limitation, financing and continuation statements, and will take all further action and will cause all further action to be taken in order to create, preserve, perfect and protect the security interest granted hereby, or to enable the Collateral Agent to exercise and enforce its rights and remedies hereunder.

(g) The Issuer will not claim any credit on, or make any deduction from, the principal or interest payable in respect of the Notes, by reason of the payment of any taxes levied or assessed upon any part of the Collateral.

(h) The Issuer will not relocate its principal executive office without providing the Collateral Agent with 90 days' prior written notice thereof and completing any such filing as may be necessary to continue the perfected security interest of the Collateral Agent in the Collateral.

(i) The Issuer will not create, incur, assume or permit to exist any lien upon any of its Assets, other than liens created by this Security Agreement or any Program Document.

(j) The Issuer will not liquidate, dissolve or change its name, amend its certificate of incorporation or by-laws, or merge with or into or consolidate with or into, or convey or otherwise dispose of all or substantially all of its assets (except as contemplated herein) to, any entity. The Issuer will not issue any stock to any person other than the Owner.

(k) The Issuer will not (i) take or permit to be taken any action that would result in the issuance and sale of the Notes, as contemplated by the Program Documents, being subject to the registration requirements of the Securities Act of 1933, as amended, or (ii) issue or sell Notes in violation of the securities laws of any state having jurisdiction.

(l) The Issuer will not declare or pay any dividend in respect of, or make any distribution in respect of or redeem or purchase any of its shares if as a result of giving effect to such declaration, payment, distribution or purchase the Issuer would fail to maintain a positive net worth.

(m) The Issuer will not make any expenditure (by long-term or operating lease or otherwise) for capital assets (both realty and personalty) except for expenditures arising out of or in connection with the exercise of its rights and performance of its obligations under or pursuant to the Program Documents.

SECTION 8. EXISTENCE AND QUALIFICATION; FORMALITIES.

(a) During the term of this Agreement, the Issuer will keep in full effect its existence, rights and franchises as a corporation under the laws of the State of Delaware (unless it becomes organized under the laws of any other State, in which case the Issuer will keep in full effect its existence, rights and franchises under the laws of such other jurisdiction) and will obtain and preserve its qualification to do business in each jurisdiction in which such qualification is or shall be necessary to protect the validity and enforceability of each Program Document and each other instrument or agreement necessary or appropriate to the proper administration of this Agreement and the transactions contemplated hereby.

(b) During the term of this Agreement, the Issuer shall observe the applicable legal requirements for the recognition of the Issuer as a legal entity separate and apart from its Affiliates, including, without limitation, the following:

(i) the Issuer shall maintain corporate records and books of account separate from those of any Affiliate;

(ii) the Issuer shall not commingle its assets and funds with those of any Affiliate;

(iii) the Issuer shall hold all appropriate meetings of its board of managers and committees thereof, keep minutes of such meetings and observe all other formalities in accordance with its governing documents and applicable law;

(iv) the Issuer shall at all times hold itself out to the public under the Issuer's own name as a legal entity separate and distinct from any Affiliate and the Swap Counterparty;

(v) the Issuer shall maintain separate financial statements from those of any Affiliate;

(vi) the Issuer shall pay its own liabilities out of its own funds;

(vii) the Issuer shall maintain an arm's length relationship with its Affiliates and the Swap Counterparty;

(viii) the Issuer shall pay the salaries of its own employees;

(ix) the Issuer shall not pledge its assets for the benefit of any other Affiliate;

(x) the Issuer shall allocate fairly and reasonably any overhead for shared office space with an Affiliate; and

(xi) the Issuer shall use separate stationery, invoices and checks from those used by any Affiliate.

SECTION 9. COLLATERAL AGENT APPOINTED ATTORNEY-IN-FACT.

(a) The Issuer shall file one or more financing or continuation statements, and amendments thereto, and shall take all such further action and execute all such further documents and instruments as may be necessary or desirable in order to create, preserve, perfect and protect the security interest granted hereby, without the signature of the Issuer where permitted by law. Whenever applicable law requires the signature of the Issuer on a document to be filed to preserve, perfect or protect the security interest granted hereby, the Issuer hereby appoints the Collateral Agent as the Issuer's attorney-in-fact, with full power of substitution, to sign the Issuer's name on any such document.

(b) The Issuer will at all times keep accurate and complete books and records with respect to the Collateral and agrees that each of the Collateral Agent, the Administrator and the Swap Counterparty shall have the right at any time and from time to time, upon not less than one Business Day's notice, to call at the Issuer's place of business during normal business hours to inspect and examine the books and records of the Issuer relating to the Collateral and to make extracts therefrom and copies thereof.

(c) The Issuer will observe and perform its obligations under each Program Document to which it is a party.

(d) The Issuer hereby irrevocably appoints the Collateral Agent the Issuer's attorney-in-fact, with full authority in the place and stead of the Issuer and in the name of the Issuer or otherwise, from time to time, to take any action, to execute any instruments and to exercise any rights, privileges, options, elections or powers of the Issuer pertaining or relating to the Collateral that the Collateral Agent may deem necessary or desirable to preserve and enforce its security interest in the Collateral and otherwise to accomplish the purposes of this Agreement. Other than as expressly set forth herein, the Collateral Agent shall not have any duty to take any such action, to execute any such instrument, to exercise any such rights, privileges, options, elections or powers or to sell or to otherwise realize upon any of the Collateral, as hereinafter authorized, and the Collateral Agent, shall not be responsible for any failure to do so or delay in so doing. All authorizations and agencies contained in this Agreement with respect to the Collateral are irrevocable and are powers coupled with an interest.

SECTION 10. <u>COLLATERAL AGENT MAY PERFORM</u>. If the Issuer fails to perform any agreement contained herein, the Collateral Agent may (but shall not be obligated to) itself perform, or cause performance of, such agreement.

SECTION 11. <u>SWAP EARLY TERMINATION EVENT; REMEDIES WITH RESPECT TO THE COLLATERAL; REALIZATION UPON COLLATERAL</u>.

(a) Upon the Collateral Agent's receipt of notice from the Swap Counterparty pursuant to the Master Swap Agreement, or if a Swap Early Termination Event shall have occurred and be continuing, the Collateral Agent will take such action as directed by the Swap Counterparty (unless a Swap Counterparty Default has occurred and is then continuing, in which case as directed by the Administrator), with respect to the Collateral, including but not limited to, the following:

(i) terminate, if applicable, on behalf of the Issuer, the Swap Transaction and if a Swap Counterparty Default has occurred, institute proceedings against the Swap Counterparty to enforce such Swap Transaction;

(ii) in connection with the termination of the Swap Transaction only, enforce the Secured Parties rights under and with respect to the Collateral including selling the Collateral in accordance with the

Asset Sale Procedures and deposit the proceeds thereof into the Collection Account; and

(iii) exercise any remedies of a secured party under the UCC and take any other appropriate action to protect and enforce the rights and remedies of the Secured Parties.

(b) The Collateral Agent shall have, with respect to the Collateral, in addition to any other rights and remedies that may be available to it at law or in equity or pursuant to this Agreement or any other contract or agreement, all rights and remedies of a secured party under any applicable version of the UCC of the relevant jurisdictions relating to the Collateral, and it is expressly agreed that if the Collateral Agent should proceed to dispose of or utilize the Collateral, or any part thereof, in accordance with the provisions of such versions of the UCC, ten days' notice by the Collateral Agent to the Issuer shall be deemed to be reasonable notice under any such provision requiring such notice. The Collateral Agent shall incur no liability as a result of the sale of the Collateral, or any part thereof, at any private or public sale or as a result of the exercise of any other rights or remedies which may be available to it except for amounts which arise out of the negligence or willful misconduct of the Collateral Agent. The Issuer hereby waives, to the extent permitted by applicable law, any claims against the Collateral Agent or any Secured Party arising by reason of the fact that the price at which Collateral may have been sold at such a private sale was less than the price which might have been obtained at a public sale or was less than the aggregate amount of the obligations secured hereunder, even if the Collateral Agent accepts the first offer received and does not offer such Collateral to more than one possible purchaser. The Collateral Agent, in connection with any exercise of any of the foregoing rights or remedies, may exercise the same without demand of performance or other demand, presentment, protest, advertisement or notice of any kind (any notice required by law referred to in this Section) to or upon the Issuer or any other Person (all and each of which demands, defenses, advertisements and notices are hereby waived). No holder of Notes shall have any right directly to enforce the security interests granted by this Agreement. Except as specifically set forth in this Agreement, no holder of Notes shall have any right to require the Collateral Agent to take or omit to take any action under this Agreement.

SECTION 12. [RESERVED].

SECTION 13. THE COLLATERAL AGENT.

13.1. Appointment. By accepting the benefits of the security interest granted herein, each Secured Party is deemed to hereby irrevocably designate and appoint CIBC as its Collateral Agent under this Agreement, and irrevocably authorize CIBC, as the Collateral Agent, to take such action on its behalf under the provisions of this Agreement and to exercise such powers and perform such duties as are expressly delegated to the Collateral Agent by the terms of this Agreement. Notwithstanding any provision to the contrary elsewhere in this Agreement, the Collateral Agent shall not have any duties or responsibilities, except those expressly set forth herein, and no implied covenants, functions, responsibilities, duties, obligations or liabilities shall be read into this Agreement or otherwise exist against the Collateral Agent. CIBC hereby accepts its appointment as Collateral Agent, subject to, and in reliance upon, the provisions of this Section 13.

13.2. Exculpatory Provisions. Neither the Collateral Agent nor any of its officers, directors, employees, agents, attorneys-in-fact or affiliates shall be liable for any action lawfully taken or omitted to be taken by it or such Person under or in connection with this Agreement *provided, however*, that no provision of this Agreement shall be construed to relieve the Collateral Agent or such Person from liability for its own negligent action, its own negligent failure to act or its own willful misconduct or a breach of its duties set forth herein.

13.3. Reliance by Collateral Agent. The Collateral Agent shall be entitled to rely, and shall be fully protected in relying in good faith, upon any writing, resolution, notice, consent, certificate, affidavit, letter, cablegram, telegram, telecopy, telex or teletype message, statement, order or other document or conversation believed by it to be genuine and correct and to have been signed, sent or made by the proper Person or Persons and upon written advice and statements of nationally recognized legal counsel, accountants and other experts selected by the Collateral Agent. The Collateral Agent shall in all cases act hereunder in accordance with the instructions of the Issuer, except to the extent such instructions are inconsistent with the terms of this Agreement, and provided that the Collateral Agent shall be fully justified in failing or refusing to take any action under this Agreement unless it shall first receive such advice or concurrence as it deems appropriate or it shall first be indemnified, to its satisfaction against any and all liability and expense which may be incurred by it by reason of taking or continuing to take any such action. The Collateral Agent may rely and shall be protected in acting or refraining from acting upon any communication authorized by this Agreement and upon any written instruction, notice, confirmation, request, direction, consent, report, certificate or other instrument, paper or document believed by the Collateral Agent to be genuine. The Collateral Agent may consult with nationally recognized counsel of its selection and the written advice of such counsel shall be full and complete authorization and protection in respect of any action taken, suffered or omitted by the Collateral Agent hereunder in good faith and in reliance thereon. The Collateral Agent shall not be required to advance, expend or risk its own funds or otherwise incur or become exposed to financial liability in the performance of its duties hereunder. The Collateral Agent may perform its duties and exercise its rights hereunder either directly or by or through agents, attorneys or independent contractors; provided that the Collateral Agent shall remain liable to the Issuer for the performance of such duties and obligations.

13.4. Resignation or Removal of Collateral Agent; Successor Collateral Agent.

(a) The Collateral Agent may resign as collateral agent upon 60 days' notice to the Issuer, the Administrator and the Noteholders subject to the terms herein. The Issuer or the Administrator on its behalf, at the direction of a majority in outstanding principal amount of the Noteholders, may remove the Collateral Agent at any time if (i) the Collateral Agent ceases to be eligible to continue as such under this Agreement, (ii) the Collateral Agent becomes insolvent, or (iii) the Collateral Agent fails to perform or observe any material term of this Agreement and shall not cure such failure within 30 days of receipt of notice of such failure. No resignation or removal of the Collateral Agent shall be effective unless and until (i) all Collateral has been liquidated and distributed pursuant to the terms hereof and Notes has been paid in full or (ii) a successor Collateral Agent is appointed pursuant to this Agreement. If the Collateral Agent resigns or is removed, the Collateral Agent shall reimburse the Issuer for any unaccrued, unearned fees which have been paid to the Collateral Agent.

(b) If no successor Collateral Agent shall have been appointed within 60 days following the date of such notice of resignation to the Issuer or the date of notice of removal to the Collateral Agent, the Collateral Agent or the Issuer may petition any Court of competent jurisdiction for the appointment of a successor Collateral Agent. If the Collateral Agent shall resign or be removed as collateral agent under this Agreement, then the Issuer, shall appoint a commercial bank having a capital and surplus of at least $50,000,000 as successor collateral agent for the Secured Parties. Upon (i) acceptance of such appointment by such successor collateral agent, and (ii) the filing of any necessary amendments to any UCC financing statements to reflect such appointment, such successor collateral agent shall succeed to the rights, powers and duties of the Collateral Agent, and the term "Collateral Agent" shall mean such successor collateral agent effective upon its appointment, and the former Collateral Agent's rights, powers and duties as Collateral Agent shall be terminated (subject to its lien securing any amounts due and owing to it hereunder), without any other or further act or deed on the part of such former Collateral Agent and the successor Collateral Agent shall be entitled to amend any

UCC financing statements and any other filings, recordation and declarations it deems advisable or necessary in connection with such termination and cancellation. After any retiring Collateral Agent's resignation hereunder as Collateral Agent, the provisions of Section 14 shall inure to its benefit as to any actions taken or omitted to be taken by it while it was Collateral Agent under this Agreement.

13.5. Power and Authority. The Collateral Agent represents and warrants that (a) it has all necessary power and authority to execute and deliver this Agreement and to perform all of its obligations under this Agreement, (b) it has all necessary power and authority to conduct its business as contemplated in this Agreement and (c) the execution, delivery and performance of this Agreement have been duly authorized by all necessary corporate action by the Collateral Agent.

13.6. Validity and Enforceability. The Collateral Agent represents and warrants that this Agreement constitutes a legal, valid, and binding obligation of the Collateral Agent, enforceable against the Collateral Agent in accordance with its terms, except as such enforceability may be limited by bankruptcy, insolvency, reorganization or other similar laws affecting the enforcement of creditors' rights generally and by general principles of equity, regardless of whether such enforceability shall be considered in a proceeding in equity or at law.

13.7. No Consents. The Collateral Agent represents and warrants that no consent, license, approval or authorization from, or registration or declaration with, any governmental body, agency or authority of the United States or Canada governing the banking or trust powers of the Collateral Agent, nor any consent, approval, waiver or notification of any creditor, lessor or other non-governmental body, agency or authority, is required in connection with the Collateral Agent's execution, delivery and performance of this Agreement, except such (if any) as have been obtained and are in full force and effect

13.8. No Change to Accounts. Without written notice to the Administrator, the Collateral Agent shall not change the account number or designation of any Account.

13.9. Subordination. The Collateral Agent hereby subordinates in favor of the Secured Parties any security interest the Collateral Agent may have, now or in the future, in the Accounts, in all property credited thereto and in all security entitlements with respect to such property, any and all statutory, regulatory, contractual or other rights now or hereafter existing in favor of the Collateral Agent over or with respect to the Accounts, all property credited thereto and all security entitlements to such property (including (i) any and all contractual rights of set-off or compensation, (ii) any and all statutory or regulatory rights of pledge, lien, set-off or compensation, (iii) any and all statutory, regulatory, contractual or other rights to put on hold, block transfers from or fail to honor instructions of the Administrator or (iv) any and all statutory or other rights to prohibit or otherwise limit the pledge, assignment, collateral assignment or granting of any type of security interest in the Accounts).

13.10. Securities Intermediary's Jurisdiction. The Collateral Agent agrees that its "securities intermediary's jurisdiction" (within the meaning of Section 8-110(e) of the UCC) is the State of New York.

SECTION 14. LIMITATION ON COLLATERAL AGENT'S DUTY IN RESPECT OF COLLATERAL.

(a) Beyond the exercise of reasonable care, the Collateral Agent shall not have any duty as to any Collateral in its possession or control or in possession or control of any agent or nominee of it, or any income thereon, or as to the grant, perfection or preservation of rights or security interest therein, or of the preservation of rights against prior parties or any other rights pertaining thereto. The Collateral Agent shall not be responsible for the payment of any taxes, charges, assessments or liens upon the Collateral or otherwise as to the maintenance of the Collateral. The Collateral Agent shall not be responsible for filing any financing or continuation

statements or recording any documents or instruments in any public office at any time or times or otherwise perfecting or maintaining the perfection of any security interest in the Collateral. The Collateral Agent shall be deemed to have exercised reasonable care in the custody of the Collateral in its possession if the Collateral is accorded treatment substantially equal to that which it accords its own property and shall not be liable or responsible for any loss or diminution in the value of any of the Collateral, by reason of the act or omission of any carrier, forwarding agency or other agent or bailee selected by the Collateral Agent in good faith.

(b) In the absence of actual knowledge of a Responsible Officer of the Collateral Agent, the Collateral Agent will not be deemed to have knowledge of any Event of Default under (and as defined in the Master Swap Agreement), Swap Counterparty Default, Asset Event or Early Termination Event unless notified thereof in writing by the Issuer or the Swap Counterparty.

SECTION 15. WAIVER OF STAYS, ETC. To the full extent that the Issuer may lawfully so agree, the Issuer agrees that it will not at any time plead, claim or take the benefit of any appraisement, valuation, stay, extension, moratorium or redemption law now or hereafter in force to prevent or delay the enforcement of this Agreement or the absolute sale of any portion of or all of the Collateral or the possession thereof by any purchaser at any sale under this Agreement, and the Issuer, for itself and all who may claim under the Issuer, as far as the Issuer now or hereafter lawfully may do so, hereby waives the benefit of all such laws.

SECTION 16. PAYMENT OF COLLATERAL AGENT FEES AND EXPENSES. Subject to Section 21, the Issuer agrees:

(a) to pay to the Collateral Agent, the fees and other expenses of the Collateral Agent, as set forth in the schedule of fees and expenses furnished by the Collateral Agent and which have been agreed to by the Issuer~~,~~ and the Manager ("Administrative Expenses");

(b) to pay or reimburse the Collateral Agent, with the consent of the Administrator for all of its reasonable out-of-pocket costs and expenses (including counsel fees and expenses) incurred in connection with (i) the preparation and execution of, and any amendment, supplement or modification to, this Agreement and any other documents prepared in connection herewith or therewith, and the consummation of the transactions contemplated hereby and thereby and (ii) its acceptance and administration of the agency hereunder and the enforcement or preservation of any rights under this Agreement and any such other documents, including, without limitation, reasonable fees and disbursements of counsel to the Collateral Agent; and

(c) to indemnify the Collateral Agent (which for purposes of this Section 16 shall include the Collateral Agent's directors, officers, employees, or agents), and hold the Collateral Agent harmless, from and against any and all losses (except the Collateral Agent's loss of profit), liabilities (including liabilities for penalties), actions, suits, judgments, demands, damages, out-of-pocket costs and expenses (including, without limitation, interest and reasonable attorneys' fees, but excluding costs and expenses attributable solely to administrative overhead) arising out of, in connection with, or resulting from, the acceptance or the exercise of the Collateral Agent's rights and/or the performance of its duties, hereunder; provided, however, that the Issuer shall not be liable to indemnify the Collateral Agent for, or hold the Collateral Agent harmless from, damage, cost or expense resulting from or attributable to the Collateral Agent's gross negligence or willful misconduct or that of its directors, officers, employees or agents. The foregoing indemnity includes, but is not limited to, any action taken or omitted to be taken by the Collateral Agent upon telephonic instructions (authorized herein) received by the Collateral

Agent from, or believed by the Collateral Agent in good faith to have been given by, the proper person or persons.

The Issuer's obligations to the Collateral Agent under this Section 17 shall survive the termination of this Agreement and the resignation or removal of the Collateral Agent.

SECTION 17. AMENDMENTS, ETC.

(a) Subject to Section 17(b), the Issuer and the Collateral Agent, with the consent of the Swap Counterparty (provided no Swap Counterparty Default has occurred and is then continuing), at any time and from time to time, may enter into one or more amendments to this Agreement for the purpose of modifying this Agreement without consent of the Secured Parties (i) to cure any error or any ambiguity or to correct or supplement any provisions therein, (ii) to add to the duties or obligations of the Issuer thereunder, (iii) to evidence and provide for the acceptance of appointment by a successor Collateral Agent, or (iv) to add any other provisions with respect to matters or questions arising under this Agreement, which provisions or supplements shall not be inconsistent with the provisions of this Agreement

(b) The Collateral Agent shall be entitled to request and receive an opinion of counsel from the Issuer stating that (i) the amendment complies with the requirements of this Agreement and (ii) the amendment is legal, valid, binding and enforceable in accordance with its terms.

(c) No amendment, modification or waiver of any provision of this Agreement shall be effective unless the same shall be in writing and signed by each party hereto. Any such amendment, modification or waiver shall be effective only in the specific instance and for the purpose for which it is given.

SECTION 18. NOTICES. Unless otherwise specified herein, all notices, requests, and other communications to any party hereunder shall be in writing (including bank wire, telecopier or similar writing) and shall be given to such party at its address or telecopier number set forth below or such other address or telex or telecopier number as such party may hereafter specify by notice to the other party. Each such notice, request or other communication shall be effective (i) if given by telecopier or other form of facsimile transmission, when the recipient receives a legible transmission thereof or (ii) if given by any other means, when delivered at the address specified in this Section:

(a) If to the Issuer:

Thermopylae Funding Corp.
c/o Global Securitization Services, LLC
114 W. 47th Street, Suite 1715
New York, NY 10036
Attention: Andrew L. Stidd
Telephone No.: (212) 302-5151
Facsimile No.: (212) 302-8767

with a copy to the Administrator

(b) If to the Administrator:

Canadian Imperial Bank of Commerce, New York Agency
425 Lexington Avenue
New York, New York 10017
Attention: John Rozario

Telephone No.: 212-856-4250
Telecopier No.: 212-856-6526

(c) If to the Swap Counterparty:

CIBC World Markets Plc
Cottons Centre
Cottons Lane
London, England SE1 2QA

Attention: Ian Howard

Telephone No.:+44-207-234-7852
Telecopier No.:+44-207-234-7472

with a copy to:

Canadian Imperial Bank of Commerce, New York Agency
425 Lexington Avenue
New York, New York 10017
Attention: Gina S. Ghent

Telephone No.: 212-856-6538
Telecopier No.: 212-856-6098

(d) If to the Collateral Agent:

Canadian Imperial Bank of Commerce, New York Agency
425 Lexington Avenue
New York, New York 10017
Attention: John Rozario

Telephone No.: 212-856-4250
Telecopier No.: 212-856-6526

SECTION 19. CONTINUING SECURITY INTEREST. This Agreement shall create a continuing security interest in the Collateral and shall (a) remain in full force and effect until the payment in full of the Obligations, (b) be binding upon the Issuer and its successors and assigns and (c) inure to the benefit of the Collateral Agent, the Secured Parties and their respective successors, transferees and assigns. Upon (i) the termination of the security interest created hereby pursuant to clause (a) above and (ii) receipt by the Collateral Agent of an officer's certificate from the Issuer stating that all conditions precedent to such termination have been complied with and requesting the release of such security interest, and if requested in writing by the Collateral Agent, an opinion of counsel as to such matters in connection with such termination as the Collateral Agent may reasonably request in writing, the Collateral Agent shall, at the Issuer's expense deliver to the Issuer a release of all security interests granted by the Issuer to the Collateral Agent pursuant to this Agreement.

SECTION 20. NO WAIVER; CUMULATIVE REMEDIES. No failure on the part of the Collateral Agent to exercise, and no delay in exercising, any right, power or remedy hereunder shall operate as a waiver thereof, nor shall any single or partial exercise of any such right, power or remedy by the Collateral Agent preclude any other or further exercise thereof or the exercise of any other right, power or remedy. All rights, powers and remedies hereunder are cumulative and are not exclusive of any other rights, powers and remedies provided by law.

SECTION 21. LIMITED RECOURSE.

(a) No Secured Party will have any recourse to the Issuer or any other assets of the Issuer other than the Collateral.

(b) The Collateral Agent agrees that all amounts payable by the Issuer to the Collateral Agent for or in connection with its services as Collateral Agent, are fully subordinated to amounts due from the Issuer to (i) the Swap Counterparty and (ii) the Noteholders in respect of the Notes and shall be paid solely out of Available Funds. If and to the extent that at any time the Available Funds are insufficient to pay any amount otherwise payable by the Issuer to the Collateral Agent at such time, such unpaid obligation shall not be payable until the earliest date on which the Available Funds are sufficient to pay such amounts and such unpaid amounts shall not constitute a "claim" (within the meaning of Section 101(5) of the Bankruptcy Code) against the Issuer.

(c) No recourse shall be had for any claims under this Agreement against any Affiliate, incorporator, shareholder, officer, or director, past, present or future, of the Issuer or of any successor corporation, either directly or through the Issuer or any successor corporation, whether by virtue of any constitution, statute or rule of law or by the enforcement of any assessment or penalty or otherwise, all such liability being, by acceptance hereof and as part of the consideration for the acceptance hereof, expressly waived and released.

SECTION 22. NO BANKRUPTCY PETITION AGAINST THE ISSUER. The Collateral Agent hereby covenants and agrees that, prior to the date which is one year and one day after the payment in full of the Notes and other Obligations, it will not institute against, or join any other Person in instituting against, the Issuer any bankruptcy, reorganization, arrangement, insolvency or liquidation proceeding or other similar proceeding under the laws of the United States or any state of the United States.

SECTION 23. JURISDICTION AND VENUE. Each party hereby irrevocably and unconditionally submits to the jurisdiction of the United States District Court for the Southern District of New York and any New York State court located in the Borough of Manhattan in New York City and of any appellate court from any thereof for the purposes of any legal suit, action or proceeding arising out of or relating to this Agreement (a "Proceeding"). Each party hereby irrevocably agrees that all claims in respect of any Proceeding may be heard and determined in such Federal or New York State court and irrevocably waives, to the fullest extent it may effectively to do so, any objection it may now or hereafter have to the laying of venue of any Proceeding in any of the aforementioned courts and the defense of an inconvenient forum to the maintenance of any Proceeding.

SECTION 24. GOVERNING LAW.

(a) THIS AGREEMENT AND ALL QUESTIONS RELATING TO VALIDITY, INTERPRETATION, PERFORMANCE AND ENFORCEMENT OF THIS AGREEMENT SHALL BE GOVERNED BY AND CONSTRUED, INTERPRETED AND ENFORCED IN ACCORDANCE WITH THE LAWS OF THE STATE OF NEW YORK, NOTWITHSTANDING ANY NEW YORK OR OTHER CONFLICT-OF-LAW PROVISIONS TO THE CONTRARY.

(b) REGARDLESS OF ANY PROVISION IN ANY OTHER AGREEMENT, FOR PURPOSES OF THE UCC, NEW YORK SHALL BE DEEMED TO BE THE JURISDICTION OF THE COLLATERAL AGENT IN ITS CAPACITY AS SECURITIES INTERMEDIARY AND THE ACCOUNTS (AS WELL AS THE SECURITIES ENTITLEMENTS (AS DEFINED IN THE UCC) RELATED THERETO) SHALL BE GOVERNED BY THE LAWS OF THE STATE OF NEW YORK.

SECTION 25. SEVERABILITY. If any one or more of the provisions contained in this Agreement or any document executed in connection herewith shall be invalid, illegal or unenforceable in any respect under any applicable law, the validity, legality and enforceability of the remaining provisions contained herein shall not in any way be affected or impaired. In the case of any such invalidity, illegality or unenforceability, the parties to the Agreement agree to use their best efforts to achieve the purpose of the invalid provision by a new legally valid stipulation, and, in lieu of such stipulation, as far as reasonably possible, effect shall be given to the intent manifested by the portion held invalid.

SECTION 26. COUNTERPARTS. This Agreement may be executed in two or more counterparts (and by different parties on separate counterparts), each of which shall be an original, but all of which together shall constitute one and the same instrument.

SECTION 27. HEADINGS. The headings in this Agreement are for purposes of reference only and shall not in any way limit or otherwise affect the meaning or interpretation of any of the terms of this Agreement.

SECTION 28. ASSIGNMENT OF RIGHTS, NOT ASSUMPTION OF DUTIES. Anything herein contained to the contrary notwithstanding:

(a) the Issuer shall remain liable under each of the Program Documents to which it is a party to the extent set forth therein to perform all of its duties and obligations thereunder to the same extent as if this Agreement had not been assigned;

(b) the exercise by the Collateral Agent of any of its rights, remedies or powers hereunder shall not release the Issuer from any of its duties or obligations under each of the Program Documents to which it is a party; and

(c) the Collateral Agent shall not have any obligation or liability under any of the Program Documents to which the Issuer is a party by reason of or arising out of this Agreement, nor shall the Collateral Agent be obligated to perform any of the obligations or duties of the Issuer thereunder or, except as expressly provided herein with respect to the Collateral Agent, to take any action to collect or enforce any claim for payment assigned hereunder or otherwise.

SECTION 29. WAIVER OF JURY TRIAL. THE PARTIES WAIVE, TO THE FULLEST EXTENT PERMITTED BY LAW, THE RIGHT TO TRIAL BY JURY IN ANY ACTION, PROCEEDING OR COUNTERCLAIM, WHETHER IN CONTRACT, TORT OR OTHERWISE, RELATING DIRECTLY OR INDIRECTLY TO THIS AGREEMENT.

SECTION 30. ASSIGNMENT. Neither party may assign all or any portion of its rights or obligations under this Agreement, (i) except, in the case of the Issuer, as otherwise contemplated by the Program Documents or (ii) unless the other party consents in writing thereto.

IN WITNESS WHEREOF, the parties hereto have caused this Security Agreement to be duly executed and delivered as of the date first above written.

THERMOPYLAE FUNDING CORP.

By:____________________________________
Authorized Signatory

CANADIAN IMPERIAL BANK OF COMMERCE,
as Collateral Agent

By:____________________________________
Authorized Signatory

SECURITY AGREEMENT

by and between

THERMOPYLAE FUNDING CORP.,
as Issuer

and

CANADIAN IMPERIAL BANK OF COMMERCE,
as Collateral Agent

FOR THE BENEFIT OF THE SECURED PARTIES,

Dated as of October 11, 2000

Annex A

[Glossary of Terms]

Without limiting the generality of this Agreement, the Assets shall be delivered to the Collateral Agent in accordance with the applicable provisions below, consistent with applicable law including applicable federal law and Articles 8 and 9 of the UCC, and such additional or alternative procedures as may hereafter become appropriate to effect the complete transfer of ownership of any such Collateral to the Collateral Agent free and clear of any adverse claims, consistent with changes in applicable law or regulations or the interpretation thereof:

(a) with respect to bankers' acceptances, commercial paper, negotiable certificates of deposit and other obligations that constitute instruments and are susceptible of physical delivery ("Physical Property"), by transfer of possession thereof to the Collateral Agent, indorsed to, or registered in the name of, the Collateral Agent or its nominee or indorsed in blank;

(b) with respect to a certificated security, (i) delivery thereof in bearer form to the Collateral Agent, or (ii) by delivery thereof in registered form to the Collateral Agent and (A) the certificate is indorsed to the Collateral Agent or in blank by effective indorsement or (B) the certificate is registered in the name of the Collateral Agent, upon original issue or registration of transfer by the issuer thereof;

(c) with respect to an uncertificated security, (i) by delivery of the uncertificated security to the Collateral Agent or (ii) the issuer thereof shall have agreed that it will comply with instructions originated by the Collateral Agent without further consent by the registered owner;

(d) with respect to any security issued by the U.S. Treasury, the Federal Home Loan Mortgage Corporation or by the Federal National Mortgage Association that is a book-entry security held through the Federal Reserve System pursuant to Federal book-entry regulations, (i) a Federal Reserve Bank by book entry credits the book-entry security to the securities account (as defined in 31 CFR Part 357) of a participant (as defined in 31 CFR Part 357) which is also a securities intermediary and (ii) the participant indicates by book entry that the book-entry security has been credited to the Collateral Agent's securities account;

(e) with respect to a security entitlement, (i) the Collateral Agent shall have become the entitlement holder or (ii) the securities intermediary shall have agreed that it will comply with entitlement orders originated by the Collateral Agent without further consent by the entitlement holder;

(f) with respect to any Asset cleared through a depositary or book-entry system organized other than under the laws of the United States or any State thereof or located in a jurisdiction other than the United States or any State thereof, by delivery by the Issuer to the Collateral Agent prior to the acquisition of such Asset an opinion of counsel (reasonably satisfactory to the Collateral Agent) and upon acquisition of such Asset by delivery of such Asset to the Collateral Agent in a manner consistent with such opinion so as to cause to Collateral Agent to have a valid, perfected, first-priority security interest in such Asset.

(g) with respect to any other Asset, by delivery to the Collateral Agent prior to the acquisition of such item of Collateral of an opinion of counsel (reasonable satisfactory to the Collateral Agent) and upon acquisition of such item of Collateral by transfer of such item of

Collateral in a manner consistent with such opinion of counsel so as to cause the Collateral Agent to have a valid, perfected, first-priority security interest in such item of Collateral;

(h) for the purpose of clauses (b) and (c) hereof "delivery" means: (i) with respect to a certificated security, (A) the Collateral Agent acquires possession thereof, (B) another person (other than a securities intermediary) either acquires possession thereof on behalf of the Collateral Agent or, having previously acquired possession thereof, acknowledges that it holds for the Collateral Agent, or (C) a securities intermediary acting on behalf of the Collateral Agent acquires possession of thereof, only if the certificate is in registered form and has been specially indorsed to the Collateral Agent by an effective endorsement; and (ii) with respect to an uncertificated security, (A) the issuer registers the Collateral Agent as the registered owner, upon original issue or registration of transfer; or (B) another person (other than a securities intermediary) either becomes the registered owner thereof on behalf of the Collateral Agent or, having previously become the registered owner, acknowledges that it holds for the Collateral Agent;

(i) except as otherwise indicated, each of the following terms shall have the meaning assigned to such term in the UCC: "certificated security", "effective indorsement", "entitlement holder", "instrument", "securities account", "security entitlement", "securities intermediary", and "uncertificated security";

(j) in each case of delivery contemplated herein, the Collateral Agent shall make appropriate notations on its records, and shall cause the same to be made on the records of its nominees, indicating that securities are held in trust pursuant to and as provided in this Agreement.